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<b>(54) Title:</b> METHOD FOR INHIBITING TUMOR ANGIOGENESIS IN A LIVING SUBJECT  <b>(57) Abstract</b>  The present invention provides a method for inhibiting tumor angiogenesis in a living subject. The method relies upon tumor angiogenesis mediated by vascular endothelial growth factor and specified induced integrin cell surface receptors expressed on the endothelial cells of tumor-included and tumor-associated blood vessels. The methodology also administers at least one antagonistic preparation effective against specified induced and expressed integrin heterodimers on the endothelial cell surface of the living subjects, the consequence of which results in an effective inhibition of tumor angiogenesis in vivo.		

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## METHOD FOR INHIBITING TUMOR ANGIOGENESIS IN A LIVING SUBJECT

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### FIELD OF THE INVENTION

The present invention is concerned with angiogenesis broadly and with tumor angiogenesis directly; and is focused on means and methods for  
10 inhibiting tumor angiogenesis involving vascular endothelial growth factor ("VEGF") and integrin heterodimer surface receptors found in the vasculature of a living subject.

### BACKGROUND OF THE INVENTION

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Angiogenesis, the formation of new capillaries and blood vessels, is a complex process first recognized in studies of wound healing and then with investigations of experimental tumors. Angiogenesis involves extracellular matrix remodeling, endothelial cell migration and proliferation, and functional  
20 maturation of endothelial cells into mature blood vessels [Brier, G. and K. Alitalo, Trends Cell Biol. 6: 454-456 (1996)]. Although the process generally has been studied for more than 50 years, the existence and in-vivo effects of several discrete angiogenic factors have been identified just over a decade ago [Folkman, J. and M. Klagsburn, Science 235: 444-447 (1985)]. Clearly,  
25 in normal living subjects, the process of angiogenesis is a normal host response to injury; and as such is an integral part of the host body's homeostatic mechanisms.

In distinction, tumor angiogenesis is the specific development in-vivo of an adequate blood supply for a solid tumor mass; and the growth of a  
30 tumor in-vivo beyond the size of a few millimeters in diameter is believed to be dependent upon the existence, maintenance, and continued development of sufficient and functional blood vasculature in-situ. In a variety of experimental tumor systems, tumor survival and growth has been linked with new capillary and new blood vessel formation. Histological examination of  
35 such neoplasms has revealed that tumor cells typically surround blood capillaries in a cylindrical configuration with a radius not exceeding about 200

micrometers - the critical travel distance for diffusion of molecular oxygen [Folkman, J., Cancer Res. 46: 467-473 (1986)]. Moreover, in the cancer patient, tumor angiogenesis originates at least in part from the sprouting of new capillaries and blood vessels directly from the pre-existing and functional normal vasculature; and possibly also from stem cells existing in the blood. Tumor angiogenesis thus involves endothelial cell penetration of the vascular basement membrane in a pre-existing blood vessel; followed by endothelial cell proliferation; and then by an invasion of the extracellular matrix surrounding the blood vessel to form a newly created vascular spout [Vernon, R. and E.H. Sage, Am. J. Pathol. 147: 873-883 (1995); Auspunk, D.H. and J. Folkman, Microvasc. Res. 14: 53-65 (1977)].

A number of different biologically active and physiologically functional molecular entities appear to be individual factors of angiogenesis. Among these are the biologically active classes of substances known as vascular endothelial growth factor and the integrin protein family of cell surface receptors. Each of these two classes will be summarily reviewed as to their conventionally known properties and functions.

#### Vascular Endothelial Growth Factor

Vascular endothelial growth factor (hereinafter "VEGF"), also known as vascular permeability factor, is a 34-45 kilodalton dimeric glycoprotein; is a cytokine; and is a potent inducer of microvascular hyperpermeability. As such, VEGF is believed to be responsible for the vascular hyperpermeability and consequent plasma protein-rich fluid accumulation that occurs in-vivo with solid tumors and ascites tumors [Senger et al., Science 219: 983-985 (1983); Dvorak et al., J. Immunol. 122: 166 (1979); Nagy et al., Biochem. Biophys. Acta. 948: 305 (1988); Senger et al., Federation Proceedings 46: 2102 (1987)]. On a molar basis, VEGF increases microvascular permeability with a potency which is typically 50,000 times that of histamine [Senger et al., Cancer Res. 50: 1774-1778 (1990)].

Vascular endothelial growth factor is also noted for its mitogenic effects on vascular endothelial cells (hereinafter "EC"). VEGF is a specific EC mitogen which stimulates endothelial cell growth and promotes angiogenesis in-vivo [Conn et al., Proc. Natl. Acad. Sci. USA 87: 2628-2632 (1990); Ferrara et al., Biochem. Biophys. Res. Comm. 161: 851-858 (1989); Gospodarowicz et al., Proc. Natl. Acad. Sci. USA 86: 7311-7315 (1989); Keck

et al., Science 264: 1309 (1989); Leung et al., Science 246: 1306 (1989); Connolly et al., J. Clin. Invest. 84: 1407-1478 (1989)]. In addition, VEGF exerts a number of other effects on endothelial cells in-vitro. These include: an increase in intracellular calcium; a stimulation of inositol triphosphate formation; a provocation of von Willebrand factor release; and a stimulation of tissue factor expression [Brock et al., Am. J. Pathol. 138: 213 (1991); Clauss et al., J. Exp. Med. 172: 1535 (1990)].

Vascular endothelial growth factor elicits potent angiogenic effects by stimulating endothelial cells through two receptor tyrosine kinases, Flt-1 and KDR/Flk-1 [Dvorak et al., Am. J. Pathol. 146: 1029-1039 (1995); Mustonen, T. and K. Alitalo, J. Cell Biol. 129: 895-898 (1996)]. Although there are potentially numerous angiogenesis factors, considerable evidence has accumulated indicating that VEGF is a cytokine of importance both for neovascularization in the medically normal adult and for development of embryonic vasculature. VEGF angiogenic activity has been demonstrated in several experimental models including the chick chorioallantoic membrane [Whiting et al., Anat. Embryol. 186: 251-257 (1992)]; rabbit ischemic hind limb [Takeshita et al., J. Clin. Invest. 93: 662-670 (1994)]; tumor xenografts in mice [Potgens et al., Biol. Chem. Hoppe. Seyler 376: 57-70 (1995); Claffey et al., Cancer Res. 56: 172-181 (1996)]; and a primate model of iris neovascularization [Tolentino et al., Arch. Ophthalmol. 114: 964-978 (1996)]. Additionally, both infusion of exogenous VEGF and overexpression of VEGF endogenously were found to induce hypervascularization of avian embryos [Drake et al., Proc. Natl. Acad. Sci. USA 92: 7657-7661 (1995); Flamme et al., Dev. Biol. 171: 399-414 (1995)].

Evidence supporting the importance of VEGF for angiogenesis generally also has come from analyses of VEGF and VEGF receptor expression. These investigations have established that elevated expression of VEGF and its receptors correlate both temporally and spatially with vascularization during embryogenesis [Millauer et al., Cell 72: 835-846 (1993); Peters et al., Proc. Natl. Acad. Sci. USA 90: 8915-8919 (1993)]; and also with the angiogenesis associated with wound healing [Brown et al., J. Exp. Med. 176: 1375-1379 (1992)]; cancer [Brown et al., Cancer Res. 53: 4727-4735 (1993)]; rheumatoid arthritis [Fava et al., J. Exp. Med. 180: 341-346 (1994)]; psoriasis [Detmar et al., J. Exp. Med. 180: 1142-1146 (1994)]; delayed-type hypersensitivity reactions [Brown et al., J. Immunol. 154: 2801-

2807 (1995)]; and proliferative retinopathies [Aiello *et al.*, *N. Eng. J. Med.* 331: 1480-1487 (1994); Pierce *et al.*, *Proc. Natl. Acad. Sci. USA* 92: 905-909 (1995)]. Thus, VEGF appears not only to promote angiogenesis in a variety of experimental systems, but also appears to be overexpressed in a diversity of settings in which neovascularization is prominent.

VEGF is typically synthesized and secreted in-vivo by a variety of cultured tumor cells, transplantable animal tumors, and many different primary and metastatic human tumors [Dvorak *et al.*, *J. Exp. Med.* 174: 1275-1278 (1991); Senger *et al.*, *Cancer Res.* 46: 5629-532 (1986); Plate *et al.*, *Nature* 359: 845-848 (1992); Brown *et al.*, *Am. J. Pathol.* 143: 1255-1262 (1993)]. Solid tumors, however, must generate a vascular stroma in order to grow beyond a minimal size [Folkman, J. and Y. Shing, *J. Biol. Chem.* 267: 10931-10934 (1992)].

VEGF today is believed able to be a central mediator of angiogenesis generally as well as of tumor angiogenesis in particular. Monoclonal antibody directed against VEGF has been shown to suppress growth and decrease the density of blood vessels in experimental tumors [Kim *et al.*, *Nature* 362: 841-844 (1993)].

It will be noted and appreciated also that many research investigations reported in the scientific and patent literature have employed antibodies raised against VEGF in order to identify and characterize the functions, properties, and attributes of the VEGF molecule in-vivo. Merely illustrating the range and variety of these investigations and published reports are the following: Preparation of specific antibodies [U.S. Patent Number 5,036,003]; use of monoclonal antibodies to suppress growth and decrease density of blood vessels in tumors [Kim *et al.*, *Nature* 362: 841-844 (1993)]; inhibition of tumor growth and metastasis by antibody to VEGF [Asano *et al.*, *Cancer Res.* 55: 5296-5301 (1995)]; inhibition of VEGF activity with specific antibodies [Sioussat *et al.*, *Arch. Biochem. Biophys.* 301: 15-20 (1993)]; the structure of solid tumors and their vasculature [Dvorak *et al.*, *Cancer Cells* 3: 77-85 (1993)]; and the distribution of VEGF in tumors and the concentration of VEGF in tumor blood vessels [Dvorak *et al.*, *J. Exp. Med.* 174: 1275-1278 (1991)]. The text of each and all of these cited publications concerning VEGF is expressly incorporated by reference herein.

### The Integrin Protein Family

Integrins are a specific family of cell surface receptors which function in-vivo as adhesive molecules for a large variety of different compounds and ligands. As a member of this specific receptor family, each integrin entity  
5 chemically is a heterodimeric glycoprotein; and is structurally composed of two different non-covalently linked protein subunits, each of the individual subunit moieties being chosen from among the alternative members forming a discrete 130-210 kilodalton "alpha" ( $\alpha$ ) subunit group and the individual members forming another distinct 95-130 kilodalton "beta" ( $\beta$ ) subunit group.  
10 The overall structure of an integrin receptor molecule generally is illustrated by Fig. A [reproduced from Hynes, R.O., Cell 48: 549-554 (1987); see also Springer, T.A., Fed. Proc. 44: 2660-2055 (1985); Hynes, R.O., Cell 69: 11-25 (1992); Ruoslahti et al., Kidney Internatl. 45: S17-S22 (1994); and INTEGRINS: Molecular and Biological Responses to the Extracellular Matrix,  
15 (Cheresh & Mecham, editors), Academic Press, 1994.

As seen in Fig. A, the alpha and beta subunits are joined in a non-covalent linkage to form a unitary whole - i.e., the heterodimer. Each subunit has a transmembrane segment (shown in Fig. A as a dark area); a small C-terminal cytoplasmic domain (shown in Fig. A as a stippled area); and a large  
20 N-terminal extracellular domain. The beta ( $\beta$ ) subunits as a group typically contain sequences of extensive intrachain disulphide bonding, including four repeated regions of a forty amino acid cysteine-rich segment (shown in Fig. A as a crosshatched area). Also, some alpha ( $\alpha$ ) subunit members of the group are cleaved posttranslationally to provide a heavy chain and a light chain  
25 linked by internal disulphide bonding to form the complete subunit entity. For a more detailed description of the integrin molecular structure, see Hynes, R.O., Cell 48: 549-554 (1987) and the references cited therein; Hynes, R.O., Cell 69: 11-25 (1992); Ruoslahti et al., Kidney Internatl. 45: S17-S22 (1994); and INTEGRINS: Molecular and Biological Responses to the Extracellular  
30 Matrix, (Cheresh & Mecham, editors), Academic Press, 1994.

It is essential to recognize also that each alpha subunit group and each beta subunit group has its own distinctive members, each of which can become non-covalently linked to more than one member of the corresponding subunit type. At present, the alpha subunit group comprises not less than  
35 fourteen (14) different entities; while the beta subunit group comprises not less than eight (8) different members. A representative listing and correlation

of the presently recognized possible combinations and permutations of individual  $\alpha$  and  $\beta$  subunits is shown by Fig. B. [reproduced in part from INTEGRINS: Molecular And Biological Responses to the Extracellular Matrix, (Cheresh & Mecham, editors), Academic Press, 1994, (preface page xii)].

5       The recognized biological role and in-vivo function of the integrin protein family are as cell surface receptors for cell-to-cell or cell-to-matrix interactions. Many of the individual integrin heterodimers comprising the family as a whole were first identified by their ability to bind with one specific ligand or matrix glycoprotein extracellularly. In this manner, the individual  
10 integrin heterodimers (each comprised of different  $\alpha$  and  $\beta$  subunits) have demonstrated a variety of unique and alternative specific binding affinities and capacities for a diverse range of singular extracellular ligands in-vivo. The conventionally known range of such extracellular ligands presently includes: laminin, collagen, fibronectin, vitronectin, epiligin, entactin,  
15 merosin, kalinin, invasin, tenascin, osteopontin, thrombospondin, adenovirus penton base, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and von Willebrand factor. A representative listing of the individual  $\alpha$  and  $\beta$  subunits composing the integrin unit with the corresponding specific bind affinity ligand is presented by Fig. C [also  
20 reproduced in part from INTEGRINS: Molecular and Biological Responses to the Extracellular Matrix, (Cheresh and Mecham, editors), Academic Press, 1994, (preface page xii)].

In addition, for the purposes of clarity and avoidance of misunderstandings or ambiguities, it is necessary to note and appreciate that.  
25 the reported research investigations of what are now recognized as integrin protein molecules were pursued by different persons working in different scientific fields for a variety of different purposes. As a unfortunate consequence of this historical development of the integrin field, a series of different and alternative titles were originally given and applied to substances  
30 thought first to be different - but which were subsequently found to be a single chemical structure and composition alone. This multiple naming and title designation occurrence was recognized in the literature very early; and a major effort was undertaken by 1987 to reconcile the various designations into a more consistent and uniform naming system, as is exemplified by Table 1  
35 below [reproduced from Hynes, R.O., Cell 48: 549-554 (1987)]. Thus, as Table 1 shows, the  $\alpha_1\beta_1$  integrin molecule was also known in 1987 as



"human very late activation protein 1 complex" or VLA-1. Similarly, the  $\alpha_2\beta_1$  integrin unit in 1987 was also alternatively titled "platelet membrane glycoprotein Ia-IIa complex" or GPIa/IIa; and as "human very late activation protein 2 complex" or VLA-2; and also as "fibroblast extracellular matrix receptor II", a misnomer of its true binding affinity (as shown by Fig. C).

5

Table 1. Members of the Integrin Receptor Family

	Subunit Molecular Weight ( $\times 10^{-3}$ )		Probable Subunit Composition	Known Ligands	Known Functions
	Nonreduced	Reduced			
Chicken integrin complex <sup>a</sup>	155/135/120	155/130/125	$\alpha_5\beta_1$ $\alpha_5\beta_1$	FN, LM, VN	Cell adhesion, cell migration Cytoskeletal connection
Fibronectin receptor	160/120	150/130	$\alpha_5\beta_1$	FN	Adhesion to fibronectin
Vitronectin receptor	160/100	135 <sup>b</sup> /115 + 25	$\alpha_v\beta_3$	VN	Adhesion to vitronectin
Glycoprotein IIb/IIIa	142/95	130 <sup>c</sup> /105 + 23	$\alpha_{IIb}\beta_3$	FN, FB, VN, VWF (TSP? Collagen?)	Platelet adhesion and aggregation
LFA-1	-	180/95	$\alpha_L\beta_2$	-	Leukocyte adhesion T lymphocyte help, lymphocyte cytotoxicity
Mac-1	-	170/95	$\alpha_M\beta_2$	C3bi	C3b receptor Monocyte and neutrophil adhesion
p150.95	-	150/95	$\alpha_X\beta_2$	C3b	Neutrophil adhesion
VLA-1	200/110	210/130	$\alpha_1\beta_1$	-	
VLA-2	150/110	165/130	$\alpha_2\beta_1$	-	
VLA-3	150/110	135/130	$\alpha_3\beta_1$	-	
VLA-4	140/110	150/130	$\alpha_4\beta_1$	-	
VLA-5	150/110	130/130	$\alpha_5\beta_1$	-	

<sup>a</sup> Also known as 140K complex, CSAT antigen, JG22 antigen.

<sup>b,c</sup> These  $\alpha$  chains consist of a heavy and a light chain held together by disulfide bonding.

The molecular weights are from different publications (see text for references) and thus may not be exactly comparable. Abbreviations for ligands are: FN, fibronectin; LM, laminin; VN, vitronectin; FB, fibrinogen; VWF, von Willebrand factor; TSP, thrombospondin; C3bi, inactivated form of C3b component of complement. The probable subunit composition of each receptor and the suggested nomenclature for  $\beta$  subunits are discussed in the text. A tentative nomenclature suggested for  $\alpha$  chains is as follows. Those with well-defined ligands are denoted by the first letter of the ligand (e.g.,  $\alpha_F$ , fibronectin;  $\alpha_V$ , vitronectin). Others are denoted by the first letter of the original cell type ( $\alpha_L$ , leukocyte;  $\alpha_M$ , macrophage) or, where no simple designation exists, by the numbers or letters used by the authors. In cases where the  $\alpha$  subunits are posttranslationally cleaved (e.g.,  $\alpha_{10}$ ,  $\alpha_V$ , and possibly  $\alpha_5$ ,  $\alpha_3$ ,  $\alpha_4$ ), it is suggested that the heavy and light chains be denoted by superscripts (e.g.,  $\alpha_{10}^H$ ,  $\alpha_{10}^L$  and  $\alpha_{10}^H$  =  $\alpha_{10}^L$ ). It is possible that some of the  $\alpha$  chains listed could be identical with others and that other  $\alpha$  chains exist. These ambiguities may necessitate future changes in nomenclature.

The integrin protein family as a whole, being cell surface receptors for specific extracellular matrix ligands, has been implicated in the processes of extracellular matrix remodeling, in endothelial cell migration, and in the function maturation of new endothelial cells into mature blood vessels - the complex process of angiogenesis generally. See for example, Hynes, R.O., *Cell* 69: 11-25 (1992); Ruoslahti *et al.*, *Kidney Internatl.* 45: S17S22 (1994); and Schwartz *et al.*, *Ann. Rev. Cell Dev. Biol.* 11: 549-599 (1995). Also published reports of targeted gene deletion of  $\alpha_5$  and  $\alpha_v$  integrin subunits in living mice apparently resulted in embryonic vascular defects [Hynes, R.O., *Develop. Biol.* 180: 402-412 (1996)]; and an antibody which broadly inhibited members of the  $\beta_1$  subunit was shown to inhibit development of the embryonic vasculature [Drake *et al.*, *Develop. Dyn.* 193: 83-91 (1992)].

In addition, other reported investigations employing a variety of different experimental models have demonstrated that an inhibition of tumor angiogenesis and of normal vasculature development can be achieved using an anti- $\alpha_v\beta_3$  blocking antibody [Brooks *et al.*, *Science* 264: 569-571 (1994); Brooks *et al.*, *Cell* 79: 1157-1164 (1994); Brooks *et al.*, *J. Clin. Invest.* 96: 1815-1822 (1995); Drake *et al.*, *J. Cell Sci.* 108: 2655-2661 (1995)]; as well as by using an anti- $\alpha_v\beta_5$  blocking antibody [Friedhandler *et al.*, *Science* 270: 1500-1502 (1995)].

The  $\beta$  subunit grouping in particular appears to have become a favored target of current research efforts. Thus, for example, cyclic peptide compounds have been developed which can inhibit  $\beta_1$  and  $\beta_2$  mediated adhesion [PCT Int. Pub. No. WO 96/40781 dated 19 December 1996]. Also, the function of the arginine-glycine-aspartic acid (RGD) amino acid sequence as a specific recognition sequence within ligands binding to  $\beta_3$  subunits has been the focus of several different recent innovations and novel peptide compounds. [PCT Int. Pub. No. WO 97/08203 dated 6 March 1997; PCT Int. Pub. No. WO 97/14716 dated 24 April 1997; see also U.S. Patent Nos. 5,192,746; 5,294,713; and 5,260,277.]

To illustrate the general state of the pertinent field and to provide a greater degree of descriptive detail generally regarding conventionally known properties, capabilities and chemical composition and structure for the alpha ( $\alpha$ ) subunit group and membership; the beta ( $\beta$ ) subunit group and membership; and the integrin protein family as a whole - the reader is directed to the following representative publications, all of which are also

- expressly incorporated by reference herein: Santoro, S.A., Cell **46**: 913-920 (1986); Mould et al., J. Biol. Chem. **265**: 4020-4024 (1989); Wagner et al., J. Cell Biol. **109**: 1321-1220 (1989); Guan, J.L. and R.O. Hynes, Cell **60**: 53-61 (1990); Staaaz et al., J. Biol. Chem. **265**: 4778-4781 (1990); Carter et al., J.
- 5 Cell Biol. **110**: 1387-1404 (1990); Wayner, E.A. and W.G. Carter, J. Cell Biol. **105**: 1873-1884 (1987); Fitzpatrick et al., The Structure and Development of Skin, (Jeffers, Scott & White, editors), McGraw-Hill Co., 1987; Davis et al., Biochem. Biophys. Res. Comm. **182**: 1025-1031 (1992); Elices, M.J. and M.E. Hemler, Proc. Natl. Acad. Sci. USA **86**: 9906-9910 (1989); Languino et al., J.
- 10 Cell Biol. **109**: 2455-2462 (1989); Takada, Y. and M.E. Hemler, J. Cell Biol. **109**: 397-407 (1989); Ignatius et al., J. Cell Biol. **111**: 709-720 (1990); Kirchhofer et al., J. Biol. Chem. **265**: 615-618 (1990); Kramer et al., J. Cell Biol. **111**: 1233-1243 (1990); Tawil et al., Biochemistry **29**: 6540-6544 (1990); Kern et al., J. Biol. Chem. **269**: 22811-22816 (1994); Briesewitz et al., J. Biol.
- 15 Chem. **268**: 2989-2996 (1993); Sriramarao et al., J. Cell Sci. **105**: 1001-1012 (1993); Gardner et al., Develop. Biol. **175**: 301-313 (1996); Wong et al., Cell Adhesion Commun. **4**: 201-221 (1996); and Mercurio A.M., Trends Cell Biol. **5**: 419-423 (1995); and Senger et al., Am. J. Path. **149**: 293-305 (1996).

In sum therefore, despite the very considerable body of presently

20 accumulated information and knowledge regarding vascular endothelial growth factor and the integrin heterodimer family, the relationships or involvements between these two classes of biologically active substances have been explored only minimally to date. Equally important, any respective role or function in-vivo conventionally known for either VEGF or the integrin

25 molecules individually has almost always focused on the properties and capabilities of each class of substance alone and without regard or attention to the possible influence of the other. This perspective and circumstance is true for angiogenesis broadly as well as for tumor angiogenesis in particular. For these reasons accordingly, were an effective and reliable method to be

30 developed for an inhibition of tumor angiogenesis which utilized and depended upon a direct and dependent relationship in-vivo between VEGF and specifically induced and expressed integrin cell surface receptors - such an inhibitory methodology would be recognized and appreciated as an unforeseen and un contemplated innovation by workers in this technical field.

### SUMMARY OF THE INVENTION

The present invention has multiple aspects and alternative definitions. A first aspect of the invention provides a method for inhibiting tumor angiogenesis mediated by vascular endothelial growth factor (VEGF) and integrin cell surface receptors expressed in the vasculature of a living subject, said method comprising the steps of:

- 5 allowing mobile VEGF secreted by a tumor mass present within the body of a living subject to become bound in-vivo to the surface of endothelial cells in a tumor-associated blood vessel;
- 10 permitting said bound VEGF to induce the expression of specified integrin heterodimers on the endothelial cell surface of the tumor-associated blood vessel in-vivo, said induced and expressed integrin heterodimers being selected from the group consisting of integrins composed of  $\alpha_1$  and  $\alpha_2$  integrin subunits; and then
- 15 administering at least one antagonistic antibody preparation effective against said induced and expressed specified integrin heterodimers on the endothelial cell surface to the living subject such that tumor angiogenesis is inhibited in-vivo, said antagonistic preparation comprising at least one antibody specific for an integrin subunit selected from the group consisting of
- 20 the  $\alpha_1$  and  $\alpha_2$  integrin subunits.

A second aspect of the invention provides an alternative method for inhibiting tumor angiogenesis mediated by vascular endothelial growth factor (VEGF) and integrin cell surface receptors expressed in the vasculature of a living subject, said alternative method comprising the steps of:

- 25 allowing mobile VEGF secreted by a tumor mass present within the body of a living subject to become bound in-vivo to the surface of endothelial cells in a tumor-included blood vessel;
- 30 permitting said bound VEGF to induce the expression of specified integrin heterodimers on the endothelial cell surface of the tumor-included blood vessel in-vivo, said induced and expressed integrin heterodimers being selected from the group consisting of integrins composed of  $\alpha_1$  and  $\alpha_2$  integrin subunits; and then
- 35 administering at least one antagonistic antibody preparation effective against said induced and expressed specified integrin heterodimers on the endothelial cell surface to the living subject such that tumor angiogenesis is

inhibited in-vivo, said antagonistic preparation comprising at least one antibody specific for an integrin subunit selected from the group consisting of the  $\alpha_1$  and  $\alpha_2$  integrin subunits.

5

### BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more easily understood and completely appreciated when taken in conjunction with the accompanying drawing, in which:

10 Fig. A is an illustration of the general structure of an integrin heterodimer functional as a cell surface receptor in-vivo;

Fig. B is an illustrative correlation showing the presently known possible combinations and permutations between alpha and beta integrin subunits;

15 Fig. C is an illustrative correlation of the different extracellular matrix ligands able to be bound in-vivo by different combinations of alpha and beta integrin subunits;

Fig. 1 is a statement of the amino acid sequence for the four major variant forms of VEGF;

20 Fig. 2 is a statement of the amino acid sequence for the  $\alpha_1$  integrin subunit;

Fig. 3 is a statement of the amino acid sequences for the  $\alpha_2$  integrin subunit;

25 Figs. 4A and 4B illustrate the qualitative results and densitometric qualities of northern analyses of integrin subunit mRNAs in human dermal endothelial cells stimulated with VEGF for up to 24 hours;

Fig. 5 illustrates integrin expression at the surface of dermal microvascular endothelial cells following stimulation with VEGF for 72 and 96 hours;

30 Figs. 6A-6E illustrate the results of ligand-cell attachment assays performed with different ligands, dermal microvascular endothelial cells, and specified integrin-blocking monoclonal antibodies;

Figs. 7A-7D illustrate the spreading of dermal microvascular endothelial cells on type I collagen gels; and

35 Figs. 8A-8D illustrate the inhibition of VEGF-driven angiogenesis in-vivo by a combination of monoclonal antibodies specific for  $\alpha_1$  and  $\alpha_2$  integrin subunits.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention is a method for inhibiting tumor angiogenesis mediated by vascular endothelial growth factor (VEGF) and specified integrin cell surface receptors induced and expressed in the vasculature of a living  
5 subject. As such, this unique inhibition methodology provides both the means and the manipulations for inhibiting new capillary and blood vessel formation effectively and reliably; and also provides multiple advantages and unforeseen benefits both to the physician/clinician as well as for the patient  
10 afflicted with a solid tumor mass in-vivo. Some of the unanticipated advantages and unforeseen benefits include the following:

1. The methodology recognizes for the first time that tumor angiogenesis, mediated in-vivo by vascular endothelial growth factor (VEGF), induces the expression of specified integrin heterodimers, namely  $\alpha_1\beta_1$  and  
15  $\alpha_1\beta_2$ , as cell surface receptors expressed on the endothelial cells lining the vasculature of the subject bearing a solid tumor. The method for inhibiting tumor angiogenesis in-vivo is thus based on this unforeseen recognition of this specific inter-relationship and on a dependence upon VEGF previously  
20 secreted by the tumor mass and its in-vivo effect as an inducing agent in order to induce the expression of these specific integrin heterodimers as a requisite forerunner of tumor angiogenesis.

2. The present methodology relies upon and utilizes the novel inducement and expression of specific integrin heterodimers comprised of either the  $\alpha_1$  or  $\alpha_2$  subunits as the basis and the mechanism of action for  
25 inhibiting tumor angiogenesis. The present invention is thus unique in its focus and in its dependence upon the new expression of integrin cell surface receptors constituted of either  $\alpha_1$  or  $\alpha_2$  subunit moieties as the specific means by which the inhibition can be routinely and reproducibly effected.

3. The present methodology is able to inhibit new capillary and  
30 new blood vessel formation both within the tumor mass itself as well as in the immediately adjacent blood vasculature surrounding the perimeter of the tumor mass itself. The method for inhibiting new blood vessel formation is effective for tumor-included blood vessels - those blood vessels and  
35 capillaries found within the perimeter edge and substance of the solid tumor mass; and also for tumor-associated blood vessels - those blood vessels lying outside the tumor but within about 0.5 millimeters distance of the tumor

mass itself. The present methodology is thus effective and functional in inhibiting both tumor-included and tumor-associated angiogenesis.

4. The present invention has been demonstrated to be effective in inhibiting tumor angiogenesis under in-vivo test conditions. As the  
5 experiments and empirical data presented hereinafter clearly evidence and show, the present methodology is effective in inhibiting new blood vessel formation in and around the tumor mass in a reproducible, reliable, and clinically verifiable manner. The present invention is thus deemed to be suitable as a therapeutic technique and clinical tool by which to treat human  
10 and animal subjects afflicted with a solid tumor mass in their bodies.

The reader is presumed to be both familiar and acquainted with the published scientific reports and the relevant patent literature regarding VEGF and the integrin molecular family, as well as their functions, their attributes, and their relationship to tumor angiogenesis. However, among this very large  
15 body of information known and accumulated to date, it is often difficult, if not impossible, to focus upon unusual features and critical observations which are the foundation of unforeseen developments and unexpected innovations within the field. A summary review of the scientific and evidentiary basis for the present invention will therefore serve the reader and provide the proper  
20 factual background and focus for recognizing the truly unique and unforeseen aspects of the present invention.

#### I. The Tumor Affected By Inhibition Of Angiogenesis

It will be recognized and recalled that tumor angiogenesis is the  
25 specific development in-vivo of an adequate blood supply for a solid tumor mass. Since the growth of a solid tumor mass in-vivo is believed to be dependent upon the existence, maintenance, and continuing development of a sufficient and functional blood supply and vasculature in-situ, the present invention's goal and objective is to inhibit and prevent the development of the  
30 blood supply required by a pre-existing tumor to survive and continue growth. Accordingly, it is the purpose of the present inhibitory methodology to prevent tumor angiogenesis.

With this objective and goal in mind, it is useful to address, identify, and characterize the tumor target which is to be deprived of an adequate  
35 blood supply for continued maintenance and growth. For purposes of the present invention, any solid tumor mass lying in any part of the body and in



any particular tissue or cell type is suitable as the intended target for inhibition of angiogenesis. It will be recalled that by definition a tumor is a neoplasm - an abnormal mass of cells typically exhibiting uncontrolled and progressive growth. Neoplasms are broadly classified into two categories: (1) according to the cell type from which they originate; and (2) according to their biologic behavior - whether they are benign or malignant. Accordingly, so long as the neoplasm is a solid mass of abnormal cells in which there is a distinct or discrete tumor matrix, stroma, and included and/or associated blood vasculature, that neoplasm is a proper and suitable target for inhibition of tumor angiogenesis using the present methodology.

It will also be recognized that the particular state of the neoplasm or tumor - so long as it is a definable solid mass - does not influence the suitability or use for the present invention. Thus, the tumor may be a "benign" neoplasm - that is, mild, favorable, or kindly (the opposite of malignant). Benign neoplasms are usually well circumscribed and are often encapsulated; and, by definition, do not invade locally and do not metastasize. In comparison, a "malignant" tumor is a neoplasm having the tendency to become clinically progressively worse and to result in the death of the subject. With neoplasms, the term "malignant" denotes the properties of tumor invasiveness and metastasis. In addition, the term "metastasis" is defined as the process by which malignant cells are disseminated from the tumor of origin (the primary tumor) to form a new growth (the secondary tumor) at a distant site; it is the discontinuous extension of a malignant neoplasm. Thus, it is a primary purpose and goal of the present invention to inhibit tumor angiogenesis both in benign and in malignant tumors generally wherever they may be found as a discrete tumor mass.

Accordingly, the present inhibitory methodology is directed to solid tumors found clinically within the living patient in-situ; and the entire broad class of human and animal solid mass tumors is deemed suitable for such therapeutic treatment wherever the tumor may be found within the body. Equally important, and especially for purposes of malignant tumors and neoplasms, the present inhibitory methodology is suitable for use with the tumor regardless of what kind, type, grade, age, size, stage, or cell origin may apply to the tumor in question. Thus, all types of primary and metastatic solid tumors can be treated in-vivo. Representative examples are breast cancer, endometrial cancer, colon cancer, lung cancer, kidney cancer, prostate

cancer, glioblastoma of the brain, malignant melanoma, Kaposi's sarcoma, and squamous cell carcinoma of the skin. For these reasons, the present method for inhibiting tumor angiogenesis is deemed to be a broadly applicable and clinically valuable therapeutic treatment.

5

## II. The Underlying Basis For The Present Inhibitory Methodology

The present invention relies on and utilizes three events as working principles. These are:

- 10 (1) Mobile vascular endothelial growth factor (VEGF) secreted by the tumor mass in-vivo functions as the initiator molecule only by becoming bound to the surface of an endothelial cell in a tumor-included or a tumor-associated blood vessel. The recognition and functional value of bound VEGF as a requisite mediator and initiator moiety for tumor angiogenesis to occur is now recognized and utilized as a necessary triggering event in-vivo;
- 15 (2) The VEGF bound in-vivo on the surface of endothelial cells of tumor-included and tumor-associated blood vessels acts as an unique inducing agent to induce the expression of new specific integrin heterodimers as cell surface receptors on the endothelial cells in-situ; and
- 20 (3) The newly induced and expressed integrin proteins now serving as cell surface receptors on the endothelial cells of tumor-included and tumor-associated blood vessels are unexpectedly integrin heterodimers comprising the  $\alpha_1$  and/or  $\alpha_2$  subunits routinely. The inducement, expression, and recognition of the  $\alpha_1$  and the  $\alpha_2$  subunits in this context has never before been appreciated or utilized for the purpose of inhibiting tumor angiogenesis.
- 25 The present invention not only identifies these events as working principles by which tumor angiogenesis proceeds in-situ; but also utilizes these singular findings as the basis of manipulations by which to control and inhibit tumor angiogenesis. The present invention thus recognizes and utilizes in a unique way the fact that VEGF and induced expression of  $\alpha_1$  and
- 30  $\alpha_2$  subunits are uniquely related and involved in a progression of events which culminate as tumor angiogenesis.

The invention recognizes also for the first time that both VEGF and integrin heterodimers comprising the  $\alpha_1$  subunit and/or  $\alpha_2$  subunits are mediators of tumor angiogenesis; and will be present as part of the

35 phenomenon of tumor angiogenesis as such. Finally, the present invention employs the fact that the newly induced and expressed  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$

integrin heterodimers present at the surface of endothelial cells of tumor-included and tumor-associated blood vessels can be antagonized using particular antagonistic agents in order to neutralize, block, and deny the functional value of these newly expressed integrin heterodimers as collagen and laminin-1 receptors specifically.

### III. The Manipulative Steps Comprising The Present Methodology

The present invention is a methodology which comprises three manipulative steps. Each of the steps comprising the inhibitory treatment reflects and recognizes the underlying principles by which tumor angiogenesis is now understood to proceed; and utilizes these principles; and controls as well as manipulates the progression of events in order to achieve an inhibition of tumor angiogenesis in an effective and reliable manner. Each of the essential manipulative steps will be described individually hereinafter.

#### Step 1: Allowing Endogenous Mobile VEGF to Become Bound In-Vivo.

First and foremost, the endogenous VEGF which becomes bound in-vivo to the surface receptors of endothelial cells of tumor-included and tumor-associated blood vessels is that tumor-secreted and initially mobile VEGF which subsequently concentrates and binds selectively to the endothelium of tumor-included and/or tumor-associated blood vessels in a far greater degree than is found in normal blood vasculature and normal organs and tissues. By definition, "tumor-included blood vasculature are those blood vessels lying within the tumor stroma and are included within the matrix substance of the solid tumor mass. In comparison, "tumor-associated blood vessels" are those blood vessels lying immediately adjacent to and within about 0.5 millimeters from the solid tumor mass and its microvasculature. Tumor-associated blood vessels include both pre-existing and those newly induced by angiogenesis. Both types provide endothelial cells ("EC") which bear surface receptors for VEGF such as Flt-1 and KDR as well as heparin-containing proteoglycans on the cell surface.

VEGF is predominantly synthesized by tumor cells and, generally to a lesser degree, by tumor-associated stromal cells. Thus, the VEGF bound in-vivo on the endothelium cell surface is primarily the result and consequence

of previously mobile VEGF that had been synthesized and secreted by the nearby tumor cells.

The entirety of the VEGF which is the inducing agent of the present methodology, is and must be solely that VEGF which becomes bound in-vivo to the endothelial cells of at least one tumor-included or tumor-associated blood vessel. Should freely circulating VEGF be present in any meaningful concentration within the blood of the living subject, such circulating and unbound VEGF is uninvolved and is unrelated to the means of action, the utility, and the purposes of the present invention. It is, therefore, an essential requirement of the present invention that the VEGF in question become bound in-vivo in each and every instance to the surface of the endothelium in a blood vessel lying either within or immediately adjacent to the solid tumor mass itself.

#### 15 The Requirement

It will be recognized and appreciated that mobile VEGF in fact can be prevented from becoming bound to the surface of endothelial cells in tumor-included and tumor-associated blood vessels in-vivo; and bound VEGF can also be prevented from serving as an inducing agent. The prevention and neutralization of effects in-vivo for mobile VEGF are described by U.S. Patent Nos. 4,456,550 and 5,036,003. In addition, the in-vivo targeting of bound VEGF function is described by Strawn *et al.*, Cancer Res. **56**: 3540-3545 (1996).

The present invention, however, does not interrupt and does not prevent the singular cellular consequences stemming from VEGF becoming bound to the surface of endothelial cells in-vivo. To the contrary, it is expressly required that the endogenous VEGF be allowed to bind to the endothelial cells of tumor-included and/or tumor-associated blood vessels; and that such bound VEGF be allowed to act in-situ as an inducing agent in order that new integrin heterodimers be synthesized and expressed at the cell surface of the endothelial cells. This requirement satisfies and is in accordance with the first and second underlying principles as described previously herein.

Structurally and chemically, endogenous VEGF is a dimeric protein which is produced in-vivo in at least four major variant forms as a result of alternative splicing of mRNA [Houck *et al.*, Mol. Endocrinol. **5**: 1806-1814

(1991); Keck et al., Science 246: 1309-1312 (1989); Leung et al., Science 246: 1306-1309 (1989); Tischer et al., Biochem. Biophys. Res. Commun. 165: 1198-1206 (1989)]. The variants of human VEGF include monomer, single strands of VEGF which are respectively 121, 165, 189, and 206 amino acid residues in length. The precise amino acid sequencing in the primary structure for the four molecular species of VEGF is shown by Fig. 1 (reproduced from Ferrara et al., Endocrine Reviews 13: 18 (1992)] wherein the identity of each individual amino acid residue in sequence is given by the single-letter code system, as conventionally known and employed routinely in this field.

It will be recognized and appreciated from the information of Fig. 1 that the different amino acid segments include omissions in some instances, particularly in the center area of the molecular structure, thereby causing the shorter length strands. In addition, it is noted and recognized that the secreted and released variants of VEGF are generally two of the four: the 121 length variant is secreted and soluble; the 165 length variant is soluble and is the prevalent form which is released. The 189 length variant and the 206 length variant are forms also synthesized and secreted by the tumor cell but are mostly retained by the extracellular matrix of the cell.

#### Step 2: Allowing The Expression Of Integrin Heterodimers Comprising $\alpha_1$ and $\alpha_2$ Subunits

It is a requisite of the present methodology that the bound VEGF be permitted to induce the expression of specified integrin heterodimers on the endothelial cell surface of the tumor-included or tumor-associated blood vessel in-vivo. It is also required that the newly induced and expressed integrin heterodimers serving as cell surface receptors be comprised of  $\alpha_1$  and/or  $\alpha_2$  subunits.

It will be recognized that this step incorporates the underlying second and third principles as described previously herein; and also specifies that the newly induced and expressed integrin heterodimers be composed of either  $\alpha_1$  and/or  $\alpha_2$  subunits as a requisite result and consequence. It will be appreciated also that the existence of inducible  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin heterodimers as a consequence of VEGF activity at the endothelial cell surface is a new finding previously unknown in this field; and also that the

induced integrin protein composition must include an alpha subunit selected from the group consisting of  $\alpha_1$  and  $\alpha_2$  subunits (primarily if not exclusively).

It will also be noted that the specified integrin subunit requirement is exact, precise, and unequivocal. The composition and amino acid sequence of the human  $\alpha_1$  subunit is given by Fig. 2 herein, which has been reproduced in part from Briesewitz *et al.*, *J. Biol. Chem.* **268**: 2989-2996 (1993). Similarly, the specific amino acid composition and sequence of the human  $\alpha_2$  integrin subunit is shown by Fig. 3 herein, which has been reproduced in part from Takada, Y. and M.E. Hemler, *J. Cell Biol.* **109**: 397-407 (1989). Moreover, as an aid in recognizing the differing characteristics and properties of the  $\alpha_1$  and  $\alpha_2$  integrin subunits as discrete compositions of matter, the reader is directed to both of these scientific publications, each of which is expressly incorporated by reference herein.

In addition, as is demonstrated and described experimentally hereinafter, the typical integrin heterodimer induced by VEGF at the endothelial cell surface is the  $\alpha_1\beta_1$  protein and the  $\alpha_2\beta_1$  protein. As shown by Fig. C herein, both of these expressed integrin heterodimers are specific receptors for collagens or laminin-1 alone. These  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin heterodimers are markedly different in their ligand binding specificities and affinities from all other  $\alpha$  subunit and  $\beta$  subunit combinations. Equally important, the  $\alpha_1$  and  $\alpha_2$  integrin subunits do not have a recognition capability for peptides or other kinds of substances carrying the RGD recognition sequence - a trait which is typical of other integrin proteins. For these reasons, the  $\alpha_1$  subunit and the  $\alpha_2$  subunit are unique and unusual even among the alpha subunit family.

### Step 3: Administering At Least One Antagonistic Antibody Preparation Against The Induced And Expressed $\alpha_1$ And $\alpha_2$ Integrin Subunits

The third and final manipulation is the administration to the subject of at least one antagonistic preparation effective against the newly induced and expressed specified integrin heterodimers on the endothelial cell surface such that tumor angiogenesis is inhibited in-vivo. For this purpose of explicitly antagonizing the induced and expressed specified integrin units in-situ, the preferred agent is a function-blocking antibody preparation comprised of monoclonal and/or polyclonal antibodies which are specific for epitopes on either or both of the  $\alpha_1$  and  $\alpha_2$  integrin subunits.

A. The function-blocking antibody antagonist

The preferred function-blocking antibody antagonist will demonstrate two characteristics: It will have the capability of binding specifically to one or more epitopes present within a spatially exposed region of the  $\alpha_1$  and/or  $\alpha_2$  integrin subunit induced and expressed in vivo. In addition, the other essential characteristic of the specific function-blocking antibody is - that upon binding to the particular alpha integrin subunit, ( $\alpha_1$  and/or  $\alpha_2$ ), functional interactions between the integrin heterodimer and its ligands (collagens and laminin-1) will be prevented. Both properties are necessary and required.

The antigenic determinants recognized by the function-blocking antibodies are provided by the amino acid residues comprising the  $\alpha_1$  or  $\alpha_2$  integrin subunits as shown by Figs. 2 and 3 respectively herein. However, this specific binding capability can be demonstrated not only by a whole intact antibody, but also by F(ab')<sub>2</sub> fragments as well as by Fab fragments derived from the whole antibody structure. It will be recalled that while the whole antibody molecule is a large bulky protein having two specific binding sites, the F(ab')<sub>2</sub> fragment represents a divalent binding fragment of the whole antibody; while the Fab binding portion is a univalent binding unit having a minimum of antibody structure. Similar smaller and genetically engineered antibody units having a specific binding capability have also been recently developed; and these entities are deemed to be equally suitable for use herein.

In addition, particular methods for preparing "humanized" antibodies have been devised. See for example, Co, M.S. and C. Queen, Nature 351: 501-502 (1993); Winter, G. and W.J. Harris, TiPs 14: 139-142 (1993); Stephens et al., Immunology 85: 668-674 (1995); Kaku et al., Eur. J. Pharmacol. 279: 115-121 (1995); and the references cited within each of these publications. Humanized antibodies offer distinct therapeutic advantages; and thus are highly preferred for clinical use because they are less likely to provoke an immune response from the patient undergoing treatment.

Other methods for preparing, isolating, and purifying each of these different antibody binding segments and units are conventionally known in the scientific literature and these techniques have been available for many

years as common knowledge in this field. The user may thus chose from among all of these different structured formats - whole antibodies, antibody subunits and antibody fragments - in picking a useful antagonistic structure having a specific binding capability for an epitope in one of the spatially exposed regions of the induced  $\alpha_1$  and/or  $\alpha_2$  integrin subunits.

In general therefore, the user has the option to chose whether the function-blocking antibody antagonist(s) is obtained from monoclonal, or polyclonal or broad antisera sources. Equally important, the user will decide whether the antibody or antibody fragments should be isolated and purified prior to use; whether they should be altered into humanized antibody form; or whether the antibody antagonist can be employed as a heterogeneous mixture of different entities and varying binding affinities, only some of which will have the requisite affinity and specific binding capability for an exposed epitope on the  $\alpha_1$  or  $\alpha_2$  integrin subunit expressed in-situ. Thus, the degree of homogeneity, purity, human compatibility, affinity, and specificity of antibodies or antibody fragments and genetically engineered subunits for one or more epitopes of the  $\alpha_1$  and  $\alpha_2$  integrins is left to the discretion and needs of the user.

## 20 Immunogens

The entirety of the  $\alpha_1$  and/or  $\alpha_2$  integrin subunits or different fragments thereof theoretically can serve as immunogens insofar as antibodies obtained with such immunogens will be evaluated and selected for their specific binding and function-blocking properties.

It will be noted and appreciated also that the range and variety of the intended sites for epitope binding within the induced and expressed  $\alpha_1$  and  $\alpha_2$  integrin subunits as a whole provides a large number of potential antigenic determinants within each permissible region spatially available for use. Thus, if one choses a peptide fragment as an immunogen, it will be recalled that a minimum of 5-7 amino acid residues (in theory) are able to be employed as a haptene in order to raise specific antibodies within a living host animal. However, longer peptide lengths of at least 10-20 residues are generally preferred. It will be noted also that the various regions in the  $\alpha_1$  or  $\alpha_2$  integrin structure (shown by Figs. 2 and 3) available for use as a source of antigenic determinants each provide far longer amino acid residue segments for this purpose. Thus, if an extended segment length of amino acid residues



were purposely employed as the immunogen, a larger number of different antigenic determinants becomes available, given the range of residue choices. Accordingly, the number of potential epitopes becomes enormous; yet each of these epitopes is a potential specific binding site for the antibody antagonist(s).

For peptide immunogens, it is intended and envisioned that at least one peptide segment of suitable length (preferably at least 10-20 residues) be chosen as the immunogen in order to provide the antigenic determinants and the production of specific antibodies using a living host animal. Once the amino acid residue length and composition has been chosen (preferably in conformity with the desired requirement of being within a spatially exposed region), the chosen antigenic or haptene segment must be prepared. Often, the desired amino acid segment can be synthetically prepared using conventionally known solid phase peptide synthesis methods [such as Merrifield, RB, J. Am. Chem. Soc. 85: 2149 (1963)]. Once synthesized, it is most desirable that the chosen segment be purified (such as by gel filtration) and desirably analyzed for content and purity (such as by sequence analysis and/or mass spectroscopy).

After its isolation or synthesis, the chosen peptide segment is typically coupled to a protein carrier to form the immunogen. Conventionally suitable protein carriers available for this purpose are available in great variety from many diverse sources. The only requirements regarding the characteristics and properties of the carrier are: first, that the protein carrier be in fact antigenic alone or in combination with the synthesized chosen amino acid residue sequence; and second, that the carrier protein be able to present the antigenic determinants of the residue sequence such that antibodies specific against the amino acid residues are produced in a living host animal. Clearly, as the experiments described hereinafter, the preferred choice of protein carrier for immunization purposes include keyhole limpet hemocyanin (KLH), coupled by glutaraldehyde (GLDH), sulfo-m-maleimidobenzo (M-hydroxysuccinimide) ester (MBS), or bisdiazobenzidine (BDB). However, any other carrier protein compatible with the host to be immunized is also suitable for use. Example of such other carrier proteins include bovine serum albumin, thyroglobulin, and the like.

Immunization procedure

All immunizations and immunization procedures are performed in the conventionally known manner described in the scientific literature. It is expected that under certain use conditions, adjuvants will be employed in combination with the prepared immunogens. Alternatively, the prepared immunogens may be used alone and be administered to the animal or human host in any manner which will initiate the production of specific antibodies.

In addition, the harvesting of polyclonal antiserum and the isolation of antibody containing sera or antibody producing cells follows the conventionally known techniques and processes for this purpose. Similarly, the preparation of hybridomas follows the best practices developed over recent years for the isolation of monoclonal antibodies [Marshak-Rothstein *et al.*, *J. Immunol.* 122: 2491 (1979)].

15 Polyclonal and monoclonal antibodies

Once obtained, the polyclonal antisera and/or monoclonal antibodies and/or genetically engineered antibodies should be evaluated and verified for their ability to bind specifically with an epitope existing within a spatially exposed region the  $\alpha_1$  or  $\alpha_2$  integrin subunits and for the capability to functionally block the abilities of the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  heterodimers to bind to collagens and laminin-1. If desired, cleavage with papain will produce two Fab fragments plus the Fc fragment; whereas cleavage of the antibodies with pepsin produces the divalent  $F(ab')_2$  fragment and the Fc' fragment - all as conventionally known.

It will be expressly understood, however, that regardless of whether the antibody binding portion represents polyclonal antisera, monoclonal antibodies, the  $F(ab')_2$  fragment, Fab fragments, humanized antibodies, or other antibody species - all of these are suitable and intended for use so long as the specific function blocking capability is demonstrated after binding to at least one epitope existing within the  $\alpha_1$  and/or  $\alpha_2$  integrin subunits induced and expressed in-vivo. It is therefore deemed to be expected that a wide variety of different immunoassay systems will be employed to demonstrate the specific binding and function-blocking capabilities required by the antibody antagonists of the present invention; and that the parameters of concentration, volume, temperature, carriers, and delivery systems can be varied extensively at will when choosing antibodies and/or antibody

fragments and subunits. The present invention therefore presumes and incorporates by reference any conventionally known immunoassay technique, procedure, protocol, or other factor or parameter - all of which may be usefully employed for the evaluation and/or preparation of a specifically binding and functionally-blocking antibody antagonist.

#### Conventionally Obtainable Examples

A number of antagonistic monoclonal antibody preparations have already been reported in the scientific literature or are now commercially sold which are specific binding and function-blocking antagonists of the  $\alpha_1$  or  $\alpha_2$  integrin subunits. Representative of such antagonistic monoclonal antibodies reported in the scientific literature are those listed within Table 2 below. In addition, representative examples of commercially prepared anti- $\alpha_1$  and anti- $\alpha_2$  integrin specific and function-blocking monoclonal antibodies (MAbs) are those sold by PharMingen Corp. as described within Table 3 below. Lastly, representative examples of specifically binding and functionally-blocking mouse monoclonal antibodies (raised against purified human  $\alpha_1$  or human  $\alpha_2$  integrin proteins) which are commercially sold by Upstate Biotechnology Corp. are listed within Table 4 below.

Table 2: Conventional Function-Blocking Monoclonal  
Antibodies Specific for  $\alpha_1$  or  $\alpha_2$  Integrin  
Subunits

	Name of <u>MAB</u>	Integrin <u>Target</u>	Published <u>Reference</u>
5			
10			
	P1H5	$\alpha_2$ subunit	Staatz et al., <u>J. Cell Biol.</u> 108: 1971-1924 (1989); Staatz et al., 15 <u>J. Biol. Chem.</u> 265: 4778-4781 (1990)
	FB12	$\alpha_1$ subunit	Fabbri et al., <u>Tissue Antigens</u> 48: 47-51 (1996)
20	5E8D9	$\alpha_1$ subunit	Lugue et al., <u>FEBS Letters</u> 346: 278-284 (1994)

Table 3\*:

A. Purified Hamster Anti-Rat/Mouse CD49a (Integrin  $\alpha_1$  chain), Function-Blocking, Monoclonal Antibody (No Azide/Low Endotoxin)

5

Product Information

Catalog Number	22620S, 2.0 mg.
Description:	Purified hamster anti-rat/mouse CD49a (Integrin $\alpha_1$ chain)
Clone:	Ha31/8
Isotope:	Armenian Hamster IgG
Contents:	Purified immunoglobulin in 10 mM phosphate buffer, pH7.2 with 150 mM NaCl (0.2 $\mu$ m filtered). No sodium azide. Endotoxin level is $\leq 0.01$ ng/ $\mu$ g of protein.

15

Specificity

The Ha31/8 antibody reacts with the 180-kDa integrin  $\alpha_1$  chain (CD49a), which is a transmembrane glycoprotein that non-covalently associates with the integrin  $\beta_1$  (CD49a/CD29 or VLA-1) complex. VLA-1 is expressed on activated T cells, smooth muscle cells, and endothelial cells; and it is a receptor for collagen and laminin.<sup>1</sup> The immunogen for the Ha31/8 clone was emulsified rat glomeruli, and the monoclonal antibody is specific for both rat and mouse CD49a.<sup>2,3</sup> It has been reported that Ha31/8 antibody can block VLA-1-mediated binding to collagen.<sup>3</sup>

20

25

References

1. Miyake, S., T. Sakurai, K. Okumura, and H. Yagita. "Identification of collagen and laminin receptor integrins on murine T lymphocytes". Eur. J. Immunol. **24**: 2000-2005 (1994).
2. PharMingen. Unpublished data.
3. Mendrick, D.L., D.M. Kelly, S.S. DuMont, and D.J. Sandstrom, "Glomerular epithelial and mesangial cells differentially modulate the binding specificities of VLA-1 and VLA-2", Lab. Invest. **72**: 367-375 (1995).

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35

Table 3 (cont'd.):

B. Purified Hamster Anti-Mouse CD49b (Integrin  $\alpha_2$  chain), Function-Blocking, Monoclonal Antibody (No Azide/Low Endotoxin)

5

Product Information

Catalog Number: 09790S, 3.0 mg  
 Description: Purified anti-mouse CD49b (Integrin  $\alpha_2$  chain)  
 10 Clone: HM $\alpha_2$   
 Isotype: Armenian Hamster IgG  
 Contents: Purified immunoglobulin in 10 mM phosphate buffer, pH 7.2 with 150 mM NaCl (0.2  $\mu$ m filtered). No sodium azide. Endotoxin level is  $\leq$  0.01 ng/ $\mu$ g of protein.

15

Specificity

The HM $\alpha_2$  antibody recognizes integrin  $\alpha_2$  chain (CD49b), the 150-kDa transmembrane glycoprotein that non-covalently associates with the integrin  $\beta_1$  subunit (Cd29) to form the integrin  $\alpha_2\beta_1$  complex known as VLA-2, which  
 20 is a receptor for collagen and laminin.<sup>1</sup> VLA-2 is expressed on some splenic CD4+T lymphocytes,<sup>1,2</sup> on intestinal intraepithelial and lamina propria lymphocytes,<sup>3</sup> NK cells,<sup>2</sup> and platelets,<sup>2</sup> but it is not on thymocytes<sup>1</sup> nor Peyer's patch, peripheral lymph nodes and mesenteric lymph nodes lymphocytes.<sup>3</sup> The expression of VLA-2 is upregulated on lymphocytes in  
 25 response to mitogens.<sup>1</sup> The HM $\alpha_2$  antibody has been reported to partially block the interaction of T-cell blasts with collagen.<sup>1,4</sup>

References

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2. PharMingen. Unpublished results.
3. Tanaka, T., Y. Ohtsuka, H. Yagita, Y. Shiratori, M. Omata, and K. Okumura, Involvement of  $\alpha_1$  and  $\alpha_4$  integrins in gut mucosal injury of graft-versus-host disease. Int. Immunol. **7**: 1183-1189 (1995).
- 35 4. Noto, K., K. Kato, K. Okumura and H. Yagita, "Identification and functional characterization of mouse CD29 with a mAb," Int. Immunol. **7**: 835-842 (1995).

40 \* Source: PharMingen Corp., San Diego, CA; Technical Data Sheets.

Table 4\*:

A. Anti-Human Integrin  $\alpha 1$  (CD49a, VLA $\alpha 1$ ), Function-Blocking, Mouse Monoclonal IgG

5

Antibody Class: IgG<sub>2a</sub> purified by protein A affinity chromatography.

Immunogen: Purified human Integrin  $\alpha 1$ .

10

Source: From hybridoma produced by fusing SP2/0 mouse myeloma cells with immunized Balb/c splenocytes, and propagated as mouse ascites.

Clone 5E8D9.

15

Formulation: Frozen liquid.

Quantity: 200 $\mu$ g/vial in 116 $\mu$ l 0.1M Tris-glycine, pH 7.4, containing 0.05% sodium azide.

20

Specificity:

Integrin  $\alpha 1$ .

Species cross-reactivity not determined.

25

References

1. Luque et al., FEBS Letter 346: 278-284 (1994).
2. Arroyo et al., J. Cell Biol. 117: 659-670 (1992).

30

B. Anti-Human Integrin  $\alpha 2$  (CD49b, VLA-2), Function-Blocking, Mouse Monoclonal IgG.

- 5 Immunogen: A549 human lung carcinoma cell line.
- Antibody Class: IgG, purified by protein G affinity chromatography.
- 10 Source: From hybridoma produced by fusing P3XAg8.653 mouse myeloma cells with immunized RBF/DnJ splenocytes, and propagated as mouse ascites.
- Clone A2-IIE10.
- 15 Species Cross-reactivity: The A2-IIE10 antibody does not recognize mouse  $\alpha 2$ . Other non-human species have not been tested.
- Quantity: 200 $\mu$ g/vial in 75 $\mu$ l 0.1M Tris-glycine, pH 7.4, containing 0.05% sodium azide.
- 20 Physical Form: Frozen solution.

References

- 25 Lee et al., Cir. Res. 76: 209-214 (1995).  
Bergelson et al., Cell Adh. & Comm. 2: 455 (1994).

- 30 \* Source: Upstate Biotechnology Corp., Lake Placid, NY; Certificates of Analysis.



#### IV. The In-Vivo Inhibition Of Tumor Angiogenesis And Its Therapeutic Treatment Potential

The consequence in-vivo of practicing the present methodology properly and completely in all its manipulative steps will provide and produce  
5 an effective inhibition of tumor angiogenesis as a clinically recognizable consequence and benefit. The present invention will provide a reliable and useful procedure for denying an adequate blood supply to solid tumors in-vivo within the body of a human or animal subject. The clinical effectiveness of the inhibition methodology has been demonstrated not only by in-vitro  
10 experiments but also unequivocally shown by the empirical data provided by in-vivo animal subjects.

#### Dosages, Modes of Administration, and Pharmaceutical Formulations

Compositions embodying the specifically binding and functionally-  
15 blocking antagonistic antibody for the present invention can be administered in any manner which preserves the function of the antibody and delivers it to the tumor site - such as intravenous, subcutaneous or other parenteral administration. The prepared antagonistic antibody can be introduced by any means or routing that inhibits tumor angiogenesis as described.

20 The dosage to be administered to any patient will vary and be dependent upon the age, overall health, and weight of the human or animal recipient; the kind of concurrent treatment, if any; the frequency of concurrent treatment; and the physician's prognosis for the patient. Generally, a range  
25 doses of antagonistic antibody from 0.1 milligrams to about 10.0 milligrams per kilogram of body weight, in twice weekly or three times weekly administrations is expected to be effective to yield the desired therapeutic result.

The duration of antagonistic antibody dose administration is expected to be continued so long as a favorable clinical result is obtained. It is  
30 believed that this treatment regimen will inhibit tumor angiogenesis in-vivo; and, in this manner, act to retard or halt the growth of the solid tumor in-situ. However, it is as yet unclear whether or not this inhibitory treatment method will provide for complete regression of tumor. For this reason especially, the treatment duration and dosage should be monitored accordingly.

35 In addition, since the antagonistic antibody preparation is typically to be given intravenously, subcutaneously, or other parenteral applications, the appropriate quantity of antibody will be prepared in sterile form; exist in single

or multiple dose formats; and typically be dispersed in a fluid carrier such as sterile physiological saline or 5% dextrose solutions commonly used with injectables.

5 V. Experimental And Empirical Data

To demonstrate the merits and value of the present invention, a series of planned experiments and empirical data are presented below. It will be expressly understood, however, that the experiments described and the results provided are merely the best evidence of the subject matter as a whole which is the invention; and that the empirical data, while limited in content, is only illustrative of the scope of the invention envisioned and claimed.

Materials and Methods:

15

Cells, Cell Culture, And VEGF Stimulation

Human dermal microvascular endothelial cells (hereinafter "EC") were isolated from neonatal foreskins as described previously [Detmar et al., J. Invest. Dermatol. 39: 2195-2225 (1990)]. Cells were grown on collagen-coated dishes in a fully supplemental endothelial cell basal medium (Clonetics, San Diego, CA) containing 20% fetal calf serum (Gibco BRL, Grand Island, NY), 50  $\mu$ M dibutyryl cyclic AMP, 1  $\mu$ g/ml hydrocortisone acetate, 100 U/ml penicillin, 100 U/ml streptomycin, and 250  $\mu$ g/ml amphotericin B (Sigma Chemical Co., St. Louis, MO).

25 Endothelial cells at passage 4 to 7 were seeded at a concentration of  $1 \times 10^4$  cells/cm<sup>2</sup> into 100 mm plastic dishes (Costar, Cambridge, MA) in fully supplemented growth medium (see above). Media were replaced every second day until the cells were confluent. For those experiments involving Northern analysis, confluent cells were shifted to EC basal medium  
30 supplemented only with 2% fetal calf serum and antibiotics 24 h prior to stimulation with VEGF. For those experiments involving stimulation with VEGF for 72 h or longer, confluent cells were shifted to this medium when VEGF was added. Recombinant human VEGF<sub>165</sub>, which is the principal VEGF isoform, was purchased from R&D Systems (Minneapolis, MN) and  
35 was added to EC cultures as indicated in the experimental descriptions. All experiments were performed at least twice with similar results.

### RNA Isolation And Northern Analyses

Total cellular RNA from EC was isolated, subjected to electrophoresis, and transferred to nylon membranes as previously described [Senger *et al.*, *Am. J. Path.* **149**: 293-305 (1996)]. <sup>32</sup>P-labeled cDNA probes were prepared as described therein using purified cDNA inserts isolated from the following: human  $\alpha_2$  integrin plasmid (clone 2.72F) and human  $\alpha_3$  integrin plasmid (clone 3.10) from the American Type Culture Collection (Rockville, MD); human  $\alpha_1$  integrin plasmid (clone 3RA), generously provided by Dr. Eugene Marcantonio (Columbia U., New York, NY); and a plasmid containing a 2.5 kb human  $\beta_1$  cDNA insert, generously provided by Dr. Larry Fitzgerald (U. Utah, Salt Lake City, UT). A purified 2.0 kb human  $\beta$ -actin cDNA was purchased from Clontech (Palo Alto, CA). Hybridizations were performed as described in Kaye *et al.* [*Proc. Natl. Acad. Sci. USA* **89**: 8542-8546 (1992)]; and autoradiograms were subjected to quantitation with a Gel Doc 1000 Imaging Densitometer (Bio-Rad Laboratories, Richmond, CA).

### Cell Surface Biotinylation And Immunoprecipitation Analyses

Surface labeling with biotin was performed essentially as described in Shaw *et al.* [*J. Biol. Chem.* **268**: 11401-11408 (1993)] except that the endothelial cells were suspended at a final concentration of  $2 \times 10^6$  cells/ml and NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) was dissolved in phosphate buffered saline (PBS) and added to the cells at a final concentration of 1 mM. The biotin labeling reaction was allowed to proceed for 30 min. at room temperature with gentle agitation to maintain the cells in suspension. Then, after washing the cells twice in PBS with 50 mM ammonium chloride to eliminate and quench the biotinylating reagent, the endothelial cells were lysed in detergent-containing immunoprecipitation buffer as described previously [Senger *et al.*, *Am. J. Path.* **149**: 293-305 (1996)]. Following extraction for 30 min. at 4°C, 1.0 ml lysates were centrifuged ( $29,000 \times g$ ) at 4°C for 30 min. To control for differences in cell recovery and/or biotinylation efficiency, equal volumes of lysates were subjected to polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore Corp., Bedford, MA). Total biotinylated protein was then visualized with chemiluminescence as described in Shaw *et al.* [*J. Biol. Chem.* **268**: 11401-11408 (1993)]. Images were captured on x-ray film and

quantitated with imaging densitometry. The differences, if any, were minor; and lysate volumes were normalized accordingly for immunoprecipitation.

Immunoprecipitation was performed as described previously [Senger et al., Am. J. Path. 149: 293-305 (1996)]. Specific rabbit polyclonal antisera (Abs) to  $\alpha_1$  integrin,  $\alpha_2$  integrin, and  $\alpha_3$  integrin subunits were purchased from Chemicon International (Temecula, CA). Rabbit polyclonal Ab to the  $\beta_1$  subunit was generously provided by Dr. Richard Hynes (MIT, Cambridge, MA). All of these polyclonal Abs were raised against synthetic peptides representing C-terminal sequences of the respective integrin subunits. Immunoprecipitates were subjected to electrophoresis; transferred to PVDF membrane; visualized with chemiluminescence; and protein bands were quantitated as described above. Biotinylated protein standards purchased from Bio-Rad included myosin (Mr 200,000),  $\beta$ -galactosidase (Mr 116,000) and phosphorylase B (Mr 97,400).

#### Cell Attachment And Cell Spreading Assays

For cell attachment assays, 96 well plates (Corning Costar Corp., Cambridge, MA) were coated with matrix proteins at a concentration of 10  $\mu$ g/ml for 1 hr followed by a coating of 100 mg/ml bovine serum albumin (Cat. #A9306, Sigma Chemical Co., St. Louis, MO) for 2 h to block the remaining protein binding sites. The coating of matrix proteins included human placental collagen I and mouse EHS laminin-1 (Life Technologies, Grand Island, NY) and human placental collagens IV and V (Collaborative Biomedical, Bedford, MA). Cultured cells were prelabeled with fluorescent Cell Tracker Dye (Molecular Probes, Eugene, OR) at a concentration of 3  $\mu$ M for 30 min. and then incubated with fresh medium for 60 min. to remove any unincorporated dye. Labeled cells were gently trypsinized and suspended in serum-free medium at  $1.5 \times 10^5$  cells/ml, mixed with antibody (see below) as indicated for 15 minutes. 100  $\mu$ l of prepared cell suspension was then added to each well. After the expiration of 45 min., the unattached cells were removed by washing; and the attached cells were quantitated with a fluorescence plate reader. Attachment of the cells to the wells coated with BSA alone were negligible. Control mouse IgG and mouse monoclonal blocking antibody specific for the human  $\beta_1$  integrin subunit (clone P4C10) were purified from control serum and P4C10 ascites (Life Technologies), respectively, using the MAPS II antibody purification kit (Bio-Rad). Purified

mouse monoclonal blocking antibodies specific for the human  $\alpha_1$  integrin subunit (clone 5E8D9) and specific for the  $\alpha_2$  integrin subunit (clone A2-IIE10) were purchased from Upstate Biotechnology (Lake Placid, NY).

To assess cell spreading on collagen I gels, Vitrogen (bovine dermal collagen I, Collagen Corp., Palo Alto, CA) was neutralized according to the manufacturer's instructions; diluted to a final concentration of 500  $\mu\text{g/ml}$  with serum-free medium; and then added to 24 well plates (500  $\mu\text{l/well}$ ). After the diluted Vitrogen had polymerized at 37°C,  $1.2 \times 10^5$  cells were added to each well containing antibodies (see above).

#### Mouse Angiogenesis Assays And Analyses of Angiogenesis Inhibition By Integrin Specific Antibodies

The assay employed was essentially as described previously by Passaniti *et al.* [*Lab. Invest.* 67: 519-528 (1992)] with the following modifications. Athymic NCr nude mice (7-8 weeks old, females) were injected subcutaneously midway on the right and left back sides with 0.25 ml Matrigel (Collaborative Biomedical, Bedford, MA) at a final concentration of 10 mg/ml together with  $2.5 \times 10^6$  VEGF-transfected SK-MEL-2 cells [Claffey *et al.*, *Cancer Res.* 56: 172-181 (1996)]. Soon after injection, the Matrigel implant solidified and persisted without apparent deterioration throughout the six day assay interval. The animals were individually treated with one of the following purified, low endotoxin ( $\leq 0.01$  ng/ $\mu\text{g}$  protein), hamster monoclonal antibodies ("MAbs", Pharmingen, San Diego, CA):  $\alpha_1$ -blocking MAb (clone Ha31/8);  $\alpha_2$ -blocking MAB (clone HA $\alpha_2$ ); or control isotype standard anti-TNP MAb (clone G235-2356). After six days, the treated animals were individually euthanized and dissected; and the excised implants were then photographed.

The excised implants together with associated skin were fixed for 60 min. in 10% formalin and embedded in paraffin. Histological sections were cut, deparaffinized, and treated with 0.1% trypsin for 30 min. at 37°C to enhance antigen availability to CD31 rat monoclonal antibody (clone MEC13.3, Pharmingen). Bound rabbit (anti-rat) secondary antibody, coupled to horseradish peroxidase (Vector Labs, Burlingame, CA), was visualized with True Blue peroxidase substrate (Kirkegaard and Perry Labs, Gaithersburg, MD). The sections were counterstained with nuclear fast red (Vector Labs). Cross-sectional diameters of new blood vessels at the implant/host interface

were measured from representative photographs; and the resulting data was expressed as average diameter size  $\pm$  standard deviation ( $n = 60$  for both groups). To determine statistical significance, the empirical data were subjected to the unpaired  $t$  test.

5

Experimental Series I: VEGF induction of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$   
expression by human dermal microvascular EC.

#### Experiment A

10 Endothelial cells were stimulated with VEGF165 (20 ng/ml) for up to 24h; and mRNAs encoding  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ , and  $\beta_1$  integrin subunits were quantitated by Northern analysis. Unstimulated cells, cultured in parallel, served as controls. The results are shown by Figs. 4A and 4B.

15 Fig. 4A shows the results of Northern analysis of integrin subunit mRNAs in human dermal microvascular EC stimulated with VEGF (20 ng/ml) for up to 24 h. Ten micrograms of local cellular RNA was loaded in each well. In comparison, Fig. 4B shows the densitometric quantitation of the Northern analyses. The signal associated with each integrin mRNA was normalized to the internal  $\beta$ -actin mRNA standard to adjust for minor differences in RNA  
20 loading.

As shown by Fig. 4, VEGF stimulation resulted in a  $> 6$ -fold induction of  $\alpha_1$  and  $\alpha_2$  mRNAs. In contrast, VEGF-stimulated cells showed no induction of  $\alpha_3$  mRNA or  $\beta_1$  mRNA, in comparison with unstimulated cells. In addition,  $\alpha_5$  mRNA was not induced by VEGF stimulation (data not shown).

25

#### Experiment B

To determine if induction of  $\alpha_1$  and  $\alpha_2$  mRNAs by VEGF translated to increased expression of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  heterodimers at the EC surface, the cells were stimulated with VEGF for 72 h or 96 h; the cell surface proteins  
30 were labeled covalently with NHS-LC-biotin; and immunoprecipitations were performed with relevant antibodies. Equal numbers of control and stimulated cells were subjected to surface biotinylation; and minor differences in cell recovery and biotinylation were controlled for quantitating incorporated biotin (see Materials and Methods). The results are illustrated by Fig. 5.

35 Fig. 5 shows integrin expression at the surface of dermal microvascular EC following stimulation with VEGF (20 ng/ml) for 72 h and

96 h. Lysates from biotinylated cells were subjected to immunoprecipitation; and the immunoprecipitates were then subjected to electrophoresis in 7.5% polyacrylamide gels under non-reducing conditions. Control cells were cultured and biotinylated in parallel. As determined by densitometry,  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  expression typically were induced 5- to 7-fold by the VEGF treatment.

Accordingly, as shown in Fig. 5, stimulation of EC with VEGF resulted in a markedly increased expression of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  at the cell surface. The induction and expression of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin units were confirmed in multiple experiments (>5); and densitometric quantitation indicated 5- to 7-fold induction for both the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins. In contrast, expression of the  $\alpha_3\beta_1$  integrin was not induced by VEGF stimulation.

#### Experimental Series II: EC Attachment Mediated By $\alpha_1\beta_1$ and $\alpha_2\beta_1$ Integrins

The  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins are known to bind collagens and laminin-1; and  $\alpha_2\beta_1$  also has been reported to bind tenascin. However, the ligand binding specificities of these integrins are not absolute and are known to differ among cell types. Therefore, these experiments tested the attachment of 72 h VEGF-stimulated microvascular EC to collagens I, IV, and V, and to laminin-1 in the presence of  $\alpha_1$ -blocking MAb and/or  $\alpha_2$ -blocking MAb in comparison with  $\beta$ -blocking MAb and control IgG. The results are illustrated by Figs. 6A-6E respectively.

Figs. 6A-6E show the results of ligand to cell attachment assays performed with dermal microvascular EC and integrin-blocking MAbs. Cultured cells were stimulated with VEGF (20 ng/ml, 72 h) prior to assay for maximal induction and expression of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin units. Substrata were coated with matrix proteins, followed by a coating of BSA to block the remaining protein binding sites. Cells were allowed to attach for 45 minutes time in serum-free medium; control IgG and specific MAbs were employed at a concentration of 10  $\mu$ g/ml.

As shown by Fig. 6, the  $\alpha_1$  MAb and  $\alpha_2$  MAb each partially blocked cell attachment to collagen I; and the two MAbs in combination together inhibited cell attachment at > 90% of cell instances. The  $\beta_1$  MAb similarly inhibited cell attachment at > 95% values. Although  $\alpha_1$  MAb and  $\beta_1$  MAb inhibited cell attachment of VEGF-stimulated cells to collagen IV, cell attachment was not inhibited by  $\alpha_2$  MAb. Also only relatively poor attachment

of VEGF-stimulated cells to collagen V was observed - an event which was inhibited most significantly by  $\alpha_1$  MAb or  $\beta_1$  MAb. Moreover, adhesion to laminin-1 was blocked by  $\alpha_1$  MAb and  $\beta_1$  MAb but little or no inhibition of cell attachment was found with the  $\alpha_2$  MAb. Finally, no inhibition of cell attachment to fibronectin was observed using either  $\alpha_1$  MAb or  $\alpha_2$  MAb. Thus, these experiments demonstrate that the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins present on the surface of VEGF-stimulated microvascular EC each were important for mediating cell attachment to collagen I; and that the  $\alpha_1\beta_1$  integrin also mediated EC attachment to collagens IV and V, and laminin-1.

Experimental Series III: VEGF-Induced Expression Of  $\alpha_1\beta_1$   
And  $\alpha_2\beta_1$ : Consequences For EC  
Interactions With Three-Dimensional  
Collagen Gels In-Vitro

Interactions between microvascular EC and three-dimensional collagen gels (i.e., polymeric collagen) are deemed to be more relevant to angiogenesis than interactions between cells and collagen-coated plastic (i.e., planar collagen). Therefore, the consequences of increased  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin unit expression for interactions between microvascular EC and polymeric collagen was investigated. For these experiments, the unstimulated control and 72 h VEGF prestimulated EC were plated on type I collagen gels in the presence of control or integrin-blocking MAbs. The results are illustrated by Figs. 7A-7D respectively.

Figs. 7A-7D show the spreading of dermal microvascular EC on type I collagen gels. The control comprised unstimulated cells which were cultured in parallel with EC prestimulated with VEGF (20 ng/ml) for 72 h. Control and VEGF prestimulated cells were plated with serum-free medium on collagen gels in the absence of VEGF. After 4 h, cells were photographed. A combination of  $\alpha_1$ -blocking MAb and  $\alpha_2$ -blocking MAb (10  $\mu$ g/ml of each) abolished cell spreading of the VEGF prestimulated cells; the control IgG (20  $\mu$ g/ml) was observed to be without effect.

As shown in Fig. 7, 72 h VEGF prestimulation promoted EC spreading on polymeric collagen as compared to unstimulated EC. Similar results were obtained with EC embedded in type I collagen (data not shown). Clearly, the addition of  $\alpha_1$ -blocking MAb in combination with  $\alpha_2$ -blocking MAb completely



inhibited spreading of the VEGF-stimulated cells. Individually, the  $\alpha_1$  MAb and  $\alpha_2$  MAb each partially inhibited cell spreading - indicating that both  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  participate in interactions between microvascular EC and polymeric collagen I (not shown).

- 5           Thus, the conclusion demonstrated and supported by the empirical data is that: (1) basal expression of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  microvascular EC is not sufficient to promote cell spreading on collagen I gels; and (2) VEGF induction of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin expression correlates with EC spreading on collagen I gels; and (3) the spreading of VEGF prestimulated EC on  
10 collagen I gels is abolished by a combination of  $\alpha_1$ -blocking MAb and  $\alpha_2$ -blocking MAb.

Experimental Series IV:   Inhibition of VEGF-Driven Angiogenesis  
In-Vivo By MAbs Which Specifically Block  
15                                    $\alpha_1$  And  $\alpha_2$

- To test directly the importance of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins for VEGF-driven angiogenesis in vivo, a mouse angiogenesis model was employed together with specific hamster monoclonal MAbs which specifically block only the murine  $\alpha_1$  or  $\alpha_2$  integrin subunits. The mouse angiogenesis model,  
20 which is a modified version of one described previously in the scientific literature [Passaniti *et al.*, *Lab. Invest.* 67: 519-528 (1992)] involves subcutaneous injection of athymic nude mice with Matrigel containing human SK-MEL-2 tumor cells stably transfected for expression of murine VEGF<sub>164</sub>. Untransfected SK-MEL-2 tumor cells are known to not provoke an angiogenic  
25 response; and therefore the angiogenic stimulus provided by the VEGF transfectants is entirely or predominantly attributable to VEGF. Furthermore, the hamster monoclonal MAbs specific for murine  $\alpha_1$  and  $\alpha_2$  integrin subunits do not recognize the respective human integrin subunits; and therefore those MAbs do not interact with the transfected SK-MEL-2 cells which provide the  
30 angiogenic stimulus.

- Procedurally and experimentally, each animal received implants by subcutaneous injection, midway on the right and left back sides on day zero. Isotype-matched control Ab (300  $\mu$ g) or a combination of  $\alpha_1$  MAb and  $\alpha_2$  MAb (150  $\mu$ g each) were administered to the individual mouse by  
35 intraperitoneal injection on days 1, 3, and 5; and 5 animals were employed in each group under test. On day 6, all animals were sacrificed and dissected;

the excised implants were photographed; and the excised tissues were fixed for histological analyses which included immunostaining for the EC marker CD31 (PECAM-1). Thus, a total of 20 implants were analyzed; 10 implants were derived from animals treated with control MAb and 10 implants were  
5 derived from animals treated with  $\alpha_1$  MAb +  $\alpha_2$  MAb. The empirical findings were highly consistent within each of the two groups, and typical examples are shown in Figs. 8A-8D respectively.

Fig. 8 illustrates the inhibition of VEGF-driven angiogenesis in vivo by a combination of  $\alpha_1$ -blocking MAb and  $\alpha_2$ -blocking MAb.

10 Figs. 8A and 8B show the Matrigel implants (M) together with overlying skin. Note the reduced density of small blood vessels associated with implant from animal treated with  $\alpha_1$  MAb +  $\alpha_2$  MAb in Fig. 8B in comparison with Fig. 8A showing an animal treated with control Ab. In contrast, the larger pre-existing blood vessels appear unaffected. Figs. 8C and 8D show the  
15 immunohistochemical staining for CD31 (blue color) which reveals that new blood vessels at the interface between the Matrigel implant (M) and host dermis (D), and in association with large nerves (N), were markedly reduced in cross-sectional area at sizes  $> 90\%$  in the  $\alpha_1$  MAb +  $\alpha_2$  MAb treated animals, in comparison with controls.

20 The evidence of Figs. 8A-8D also clearly demonstrate that the overlying skin adjacent to the implants showed substantially reduced numbers of small blood vessels in the  $\alpha_1$  MAb +  $\alpha_2$  MAb treatment group in comparison with the control group (Figs. 8A and 8B). Moreover, no detectable effects of MAb on the pre-existing blood vessels were observed.  
25 Consistent with these observations, the immunohistochemical staining for CD31 demonstrated that the average cross-sectional diameter of new blood vessels lying adjacent to the angiogenic stimulus was significantly ( $p < .001$ ) reduced in size to  $8.4 \pm 1.5 \mu\text{m}$  in the  $\alpha_1$  MAb +  $\alpha_2$  MAb treatment group, in comparison with average diameter sizes of  $31.6 \pm 4.3 \mu\text{m}$  in the control MAb  
30 group (Figs. 8C and 8D). This significant reduction in average blood vessel diameter size translated into a  $> 90\%$  decrease in average cross-sectional area. Thus, the empirical results of these experiments are probative evidence that a combination of  $\alpha_1$ -blocking MAbs and  $\alpha_2$ -blocking MAbs  
35 adversely affects VEGF-driven tumor angiogenesis in vivo without detectable adverse effects on the pre-existing vasculature.

## Summary Of Experimental Data:

The experiment described herein and the empirical findings reported here indicate that VEGF potently induces expression of specific  $\alpha_1$  and  $\alpha_2$  integrin subunits by microvascular EC. To summarize, the data show that:

- 5       (a)    VEGF induces a 5- to 7-fold increase in dermal microvascular EC expression of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin heterodimers;
  - (b)    on these microvascular EC,  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  both serve as receptors for collagen I and  $\alpha_1\beta_1$  serves additionally as a receptor for collagen IV, collagen V, and laminin-1;
  - 10       (c)    VEGF induction of  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  promoted EC spreading on collagen I gels in vitro; and
  - (d)     $\alpha_1$ -blocking and  $\alpha_2$ -blocking monoclonal antibodies, in combination, markedly inhibit VEGF-driven tumor angiogenesis in vivo.
- Thus, the data indicate and reveal not only that VEGF induces  $\alpha_1\beta_1$  and
- 15        $\alpha_2\beta_1$  integrin heterodimers and collagen receptor expression by EC but also that  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrin receptor function is critical for VEGF-driven tumor angiogenesis.

The present invention is not to be limited in scope nor restricted in form except by the claims appended hereto.

What we claim is:

1. A method for inhibiting tumor angiogenesis mediated by vascular endothelial growth factor (VEGF) and integrin cell surface receptors expressed in the vasculature of a living subject, said method comprising the steps of:

allowing mobile VEGF secreted by a tumor mass present within the body of a living subject to become bound in-vivo to the surface of endothelial cells in a tumor-associated blood vessel;

permitting said bound VEGF to induce the expression of specified integrin heterodimers on the endothelial cell surface of the tumor-associated blood vessel in-vivo, said induced and expressed integrin heterodimers being selected from the group consisting of integrins composed of  $\alpha_1$  and  $\alpha_2$  integrin subunits; and then

administering at least one antagonistic antibody preparation effective against said induced and expressed specified integrin heterodimers on the endothelial cell surface to the living subject such that tumor angiogenesis is inhibited in-vivo, said antagonistic preparation comprising at least one antibody specific for an integrin subunit selected from the group consisting of the  $\alpha_1$  and  $\alpha_2$  integrin subunits.

2. A method for inhibiting tumor angiogenesis mediated by vascular endothelial growth factor (VEGF) and integrin cell surface receptors expressed in the vasculature of a living subject, said alternative method comprising the steps of:

allowing mobile VEGF secreted by a tumor mass present within the body of a living subject to become bound in-vivo to the surface of endothelial cells in a tumor-included blood vessel;

permitting said bound VEGF to induce the expression of specified integrin heterodimers on the endothelial cell surface of the tumor-included blood vessel in-vivo, said induced and expressed integrin heterodimers being selected from the group consisting of integrins composed of  $\alpha_1$  and  $\alpha_2$  integrin subunits; and then

administering at least one antagonistic antibody preparation effective against said induced and expressed specified integrin heterodimers on the endothelial cell surface to the living subject such that tumor angiogenesis is inhibited in-vivo, said antagonistic preparation comprising at least one

antibody specific for an integrin subunit selected from the group consisting of the  $\alpha_1$  and  $\alpha_2$  integrin subunits.

3. A method for inhibiting tumor angiogenesis as recited in claim 1 and 2 wherein said antagonistic antibody preparation is a monoclonal antibody.

4. The method for inhibiting tumor angiogenesis as recited in claim 1 or 2 wherein said antagonistic antibody preparation is a polyclonal antibody preparation.

5. The method for inhibiting tumor angiogenesis as recited in claim 1 or 2 wherein said antagonistic antibody preparation includes at least one entity selected from the group consisting of Fab, F(ab')<sub>2</sub>, and humanized, and genetically engineered antibody fragments.

6. The method for inhibiting tumor angiogenesis as recited in claim 1 or 2 wherein said antagonistic preparation further comprises a mixture of antibodies collectively specific for the  $\alpha_1$  and  $\alpha_2$  integrin subunits.

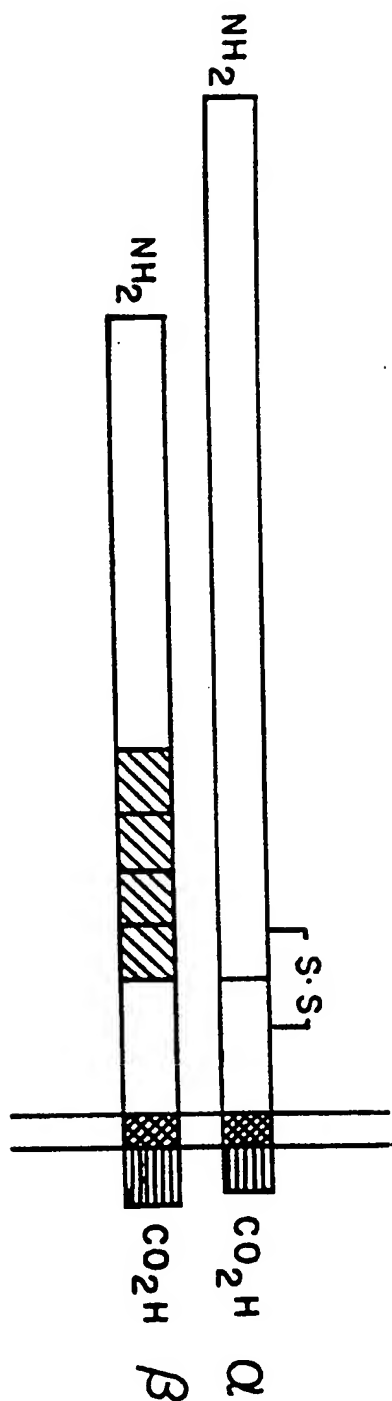
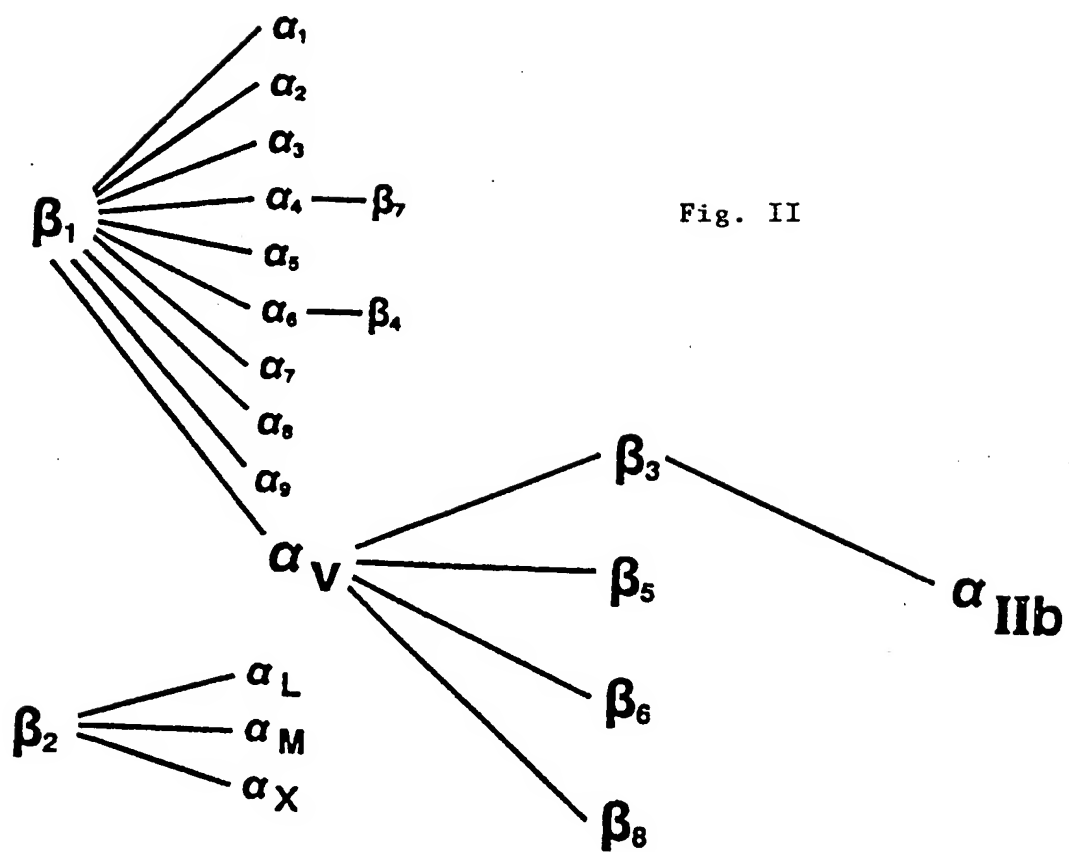
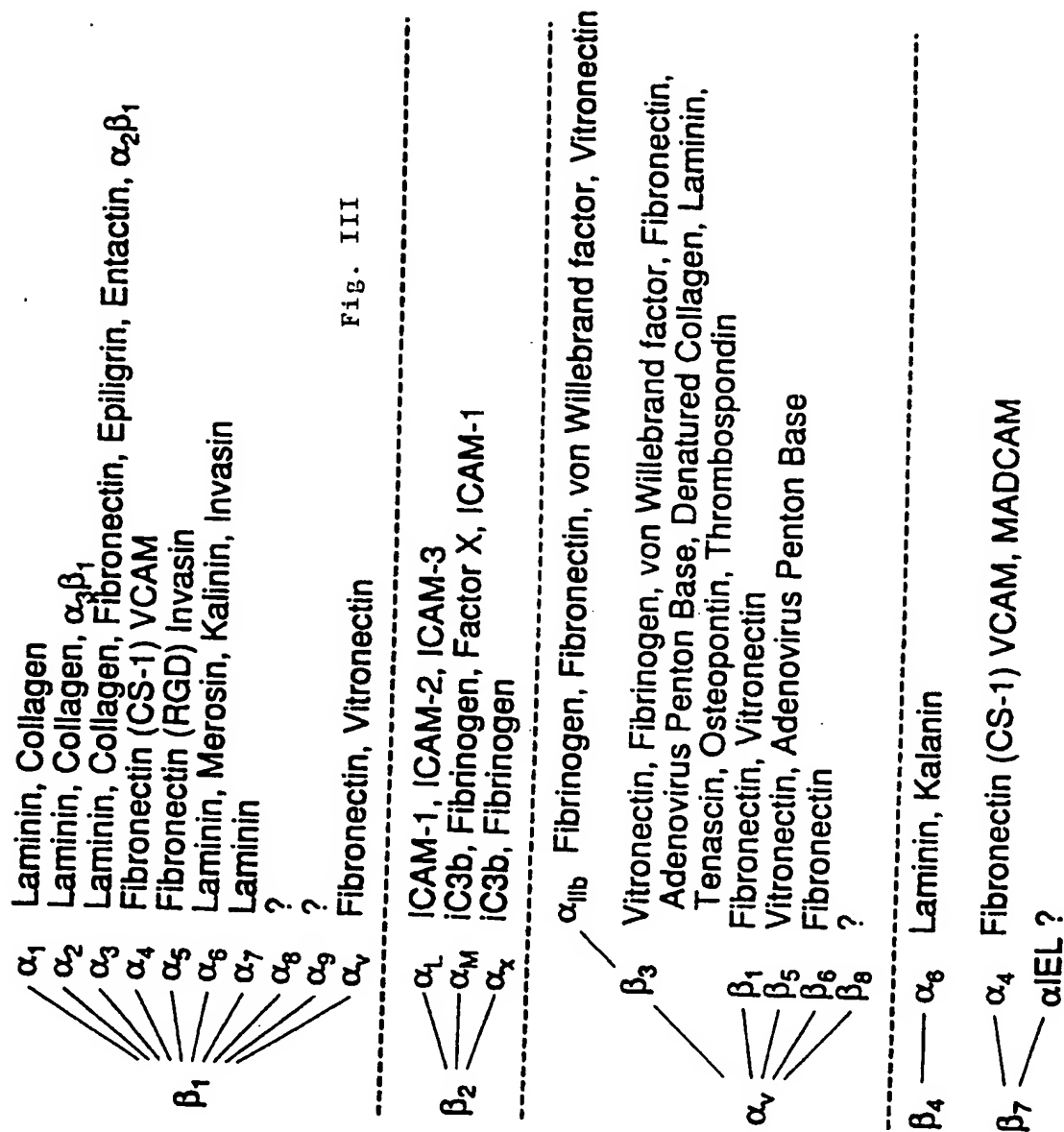


Fig. 1

2/18







VEGF	121	-26	MNFLLSWVHWSLALLYLHHAKWSQAAPMAEGGGONHHEVVKFMDVYORS
VEGF	165	-26	MNFLLSWVHWSLALLYLHHAKWSQAAPMAEGGGONHHEVVKFMDVYORS
VEGF	189	-26	MNFLLSWVHWSLALLYLHHAKWSQAAPMAEGGGONHHEVVKFMDVYORS
VEGF	206	-26	MNFLLSWVHWSLALLYLHHAKWSQAAPMAEGGGONHHEVVKFMDVYORS
VEGF	121	25	YCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTES
VEGF	165	25	YCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTES
VEGF	189	25	YCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTES
VEGF	206	25	YCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTES
VEGF	121	75	WITMQIMRIKPHOGOHIGEMSFLQHNKCECRPPKDRARGEK-----
VEGF	165	75	WITMQIMRIKPHOGOHIGEMSFLQHNKCECRPPKDRARGEK-----
VEGF	189	75	WITMQIMRIKPHOGOHIGEMSFLQHNKCECRPPKDRARGEKSVRGKGGR
VEGF	204	75	WITMQIMRIKPHOGOHIGEMSFLQHNKCECRPPKDRARGEKSVRGKGGR

FIG. 1A

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VEGF <sub>121</sub>	-----	
VEGF <sub>165</sub>	-----	PCGPCSERRRKHLFVODPO
VEGF <sub>189</sub>	125 QRRKRKKSRYKWSV-----	PCGPCSERRRKHLFVODPO
VEGF <sub>204</sub>	125 QRRKRKKS RYKSWSVYVGARCCLPWVSLPGPHPCGPCSERRRKHLFVODPO	175 TCKCCKNTDSRCKAROLELNERTCRCDKPRR
VEGF <sub>121</sub>	-----	CDKPRR
VEGF <sub>165</sub>	134 TCKCCKNTDSRCKAROLELNERTCRCDKPRR	
VEGF <sub>189</sub>	158 TCKCCKNTDSRCKAROLELNERTCRCDKPRR	
VEGF <sub>204</sub>		

FIG. 1B

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MVPRRPASLEVTVTACIWLLTVILGFCVSFNVD  
VKNSMTFSGPVEDMFGYTVQQYENEEGKWV LIGS  
PLVGQPK N\*RTGDVYKCPVGRGESLPCVKLDLPVN\*  
TSIPN\*VTEVKEN\*MTFGSTLVTPNPGGFLACGPLY  
AYRCGHLHYTTGICSDVSPTFQVVNSIAPVQEC\*S  
TOLDIVIVLDGSNSIYPMDSVTA LNDLLKRMDI  
GPKOTOVGIVOYGEN\*VTHEFNLNKYSSTEELVA  
AKKIVORGGROTM TALGTD TARKEAFTEARGARR  
GVKKVMVIVTDGEHDONHRLKKVIGDCEDENIOR  
FSIALGSYNRGN\*LSTEKFVEEIKSIASEPTKS  
FFN\*VSDELALVTIVKTLGERIFALEATADOSAAS

FIG. 2A

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FEMMSQTGFSAHYSQDMVWLGA VGAYDWN\*GT VV  
NQKASQIIIPRN\*TTFNVESTKKNEPLASYLG YTV  
NSATASSG DVL YIAGQPRYN\*HTGQV IIRMEEGN  
IKILQTLSGEOIGSYFGSILTTT DDIDKDSNTDIL  
LVGAPMYMGTEKEEGK VYVYALN\*QTRFEYQMSL  
APMEPIKQTCSSRQHNSCTTENKNEPCGARFGTAIA  
AVKDLN LDGFNDIVIGAPLEDDHGGAVYIYHSGS  
KTIRKEYAORIPSGDGKTLKFFGQSIHGEMDLN  
GDGLTDV TICGLGGAALFWSRDVAVVKVTMNFEP  
NKVNIQKKNCHMEGKETVCIN\*ATVCFEV KLKSKE  
DTIYEADLQYRVTLDSL RQISR SFFSGTQERKVQ  
RN\*ITVRKSECTKHSFYMLDKHDFQDSVRITLDFN\*

FIG. 2B

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LTDPENGPVLDDSLPSVHEYIPFAKDCGNKEKC  
ISDLSLHVATTEKDLLIVRSQNDKFN\*VSLTVKNT  
KDSAYNTRTIVHSPNLVFS GIEAIQKDSCESENH  
N\*ITCKVGYPFLRRGEMVTFKILFQFN\*TSYLMEN\*V  
TIYLSATSDSEEPP ETLSDNVVN\*ISIPVKYEVGL  
QFYSSASEYHISIAA\*ETVPEVIN\*STEDIGNEIN  
IFYLIRKSGSPMPPELKLSISFPN\*MTSNGYPVLY  
PTGLSSSENANCRPHIFEDPFSINSGKKMTT STD  
HLKRGITLDCNTCKFATITCN\*LTSSDISOVN\*VSL  
ILWKPTFIKSYFSSLN\*LTIRGELRSEN\*ASLVLS  
SNQKRELAIQISKDGLPGRVPLWVILLSAFAGLL  
LLMLLILALWKIGFFKRPLKKKMEK

FIG. 2C

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MGPERTGAAPLPLLLVLA	-12
LSQGILNCCLAYNVGLPEAKIFSGPSSEQFGYAV	23
QQFINPKGNWLLVGSPWSGFPENRMGDVYKCPVD	57
LSTATCEKLNLTSTSTIPN*VTEMKTN*MSLGLILT	91
RNMGTTGGFLTCGPLWAQQCGNQYYTTTGVCSDISP	125
DFOLSASFSPATOPCPSLIDVVVVCDESNSIYPW	159
DAVKNFLEKFVQGLDIGPTKTQVGLIQYANNPRV	193
VFNLNTYKTKEEMIVATSQTSQYGGDLTNTFGAI	227
QYARKYAYSAAASGGRRSATKVMVVVTDGESHDGS	261
MLKAVIDQCNHDNLRFGIAVLGYLNRNALDTKMN	295
LIKEIKAIASIPTERYFFN*VSDEAAALIEKAGTLG	329
EQIFSIEGTVOGGDNFOMEMSQVGFSADYS SQND	363
ILMLGAVGAFGWSGTIVQKTSHGHLIFPKQAFDQ	397

FIG.3A

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IQDRN*HSSYLGYSVAAISTGESTHFVAGAPRAN*	431
VTGQIVLYSVNENGNTVIAHRGDQIGSYFGSV	465
LCSVDVDDKDTITDVLLVGAPMYMSDLKKEGRVY	499
LFTIKKILGQHQFLEGPEGIENTRFGSAIALS	533
DINMDGFFNDVIVGSPLENQNSGAVYIYNGHQGTI	567
RTKYSQKILGSDGAFRSHLQYFGRSLDGYGDLNG	601
DSITDVSIGAFGQVVQLWSQSIADV AIEASFTPE	635
KITLVNKNAQIILKLCFSAKFRPTKQNNQVAIVY	669
N*ITLDADGFSSRVTSRGLFKENNERCLQKNMVVN	703
QAQSCPEHIIYIQEPSDVVNSLDDL RVDISLENG	737
TSPALEAYSETAKVFSIPFHKDCGEDGLCISDLV	771
LQDVRIPAAQEPPFIVSNQNKRLTFSVTLKNKRE	805

FIG. 3B

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SAYNTGIVVDFSENLFASFSLPVDGTEVTCQVA	839
ASQKSVACDVGYPAKREQQVTFITINFDNLQNL	873
QNQASLSFQALSESQEENKADNLVNLKIPLLIDA	907
EIHLTRSTNINFYEISSDGNVPSIVHSFEDVGPK	941
FIFSLKVTTGSPVPSMATVIIHIPQYTKKKNPLM	975
YLTGVQTDKAGDICCNAIDINPLKIGQTSSSVSFK	1009
SENFRTKELNCRITASCSN*VTCWLKD VHKMG EYF	1043
VN*VTTRIWN*GTFFASSTFQTVQLTAAAEINTYNPE	1077
IYVIEDNTVTIPLMIMKPKDEKAEVPTGVIIGSII	1111
AGILLLLALVAILWKLGLGFFKRKYEKMTKKNPDEID	1145
ETTELSS	1152

FIG. 3C



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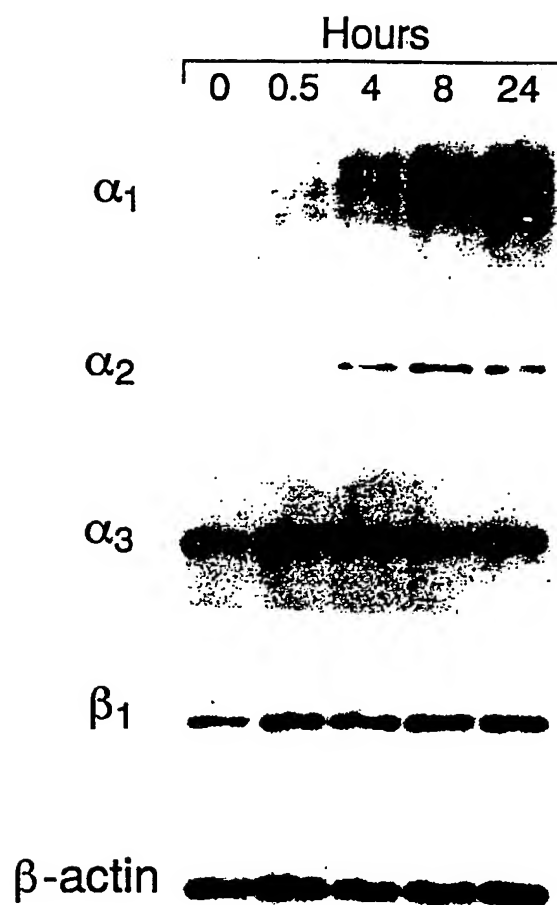


Fig. 4A

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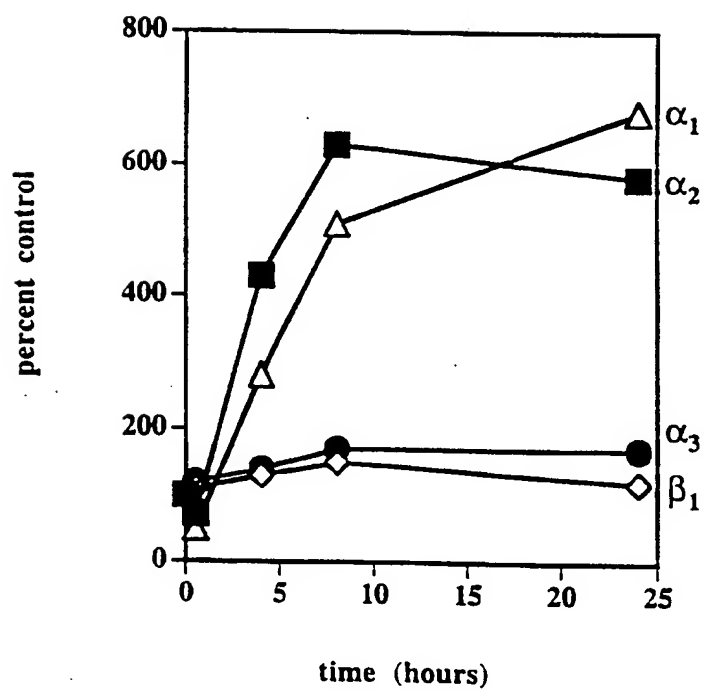


Fig. 4B

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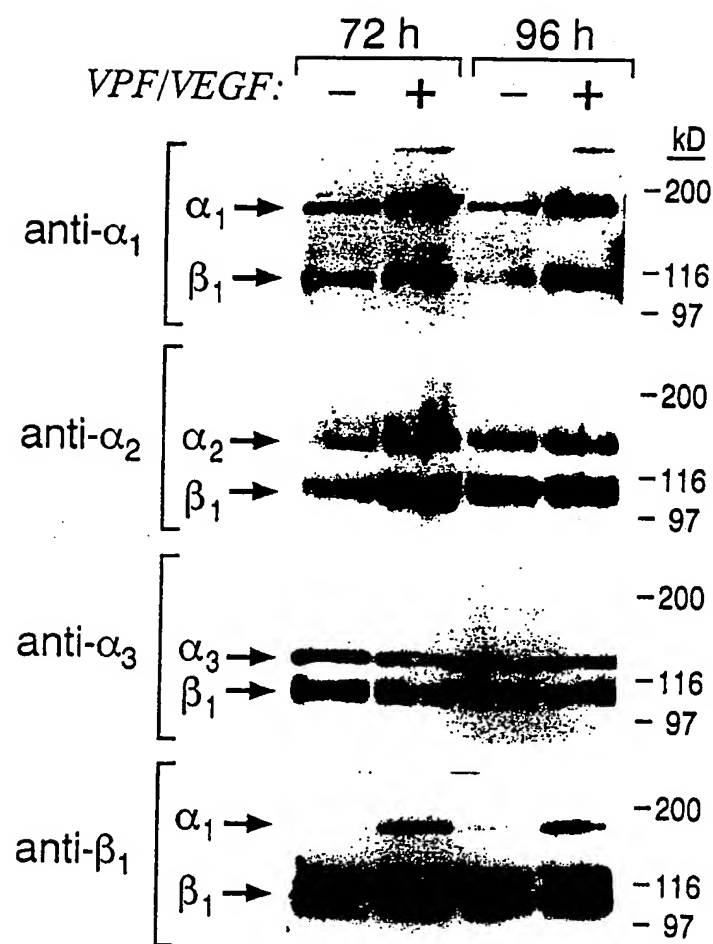


Fig. 5

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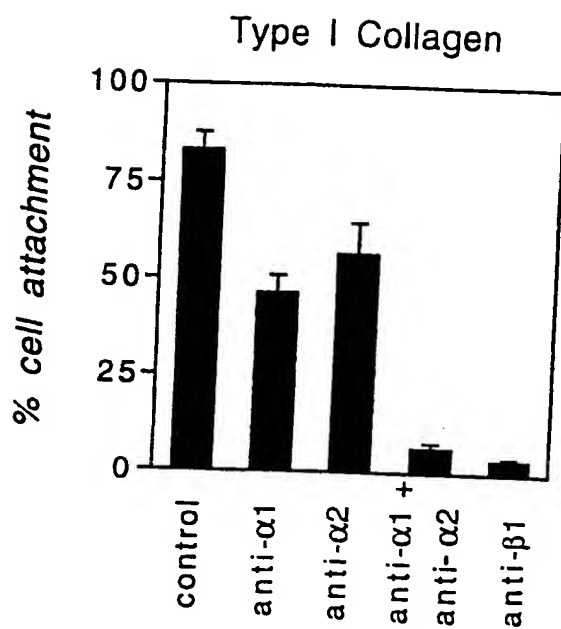


Fig. 6A

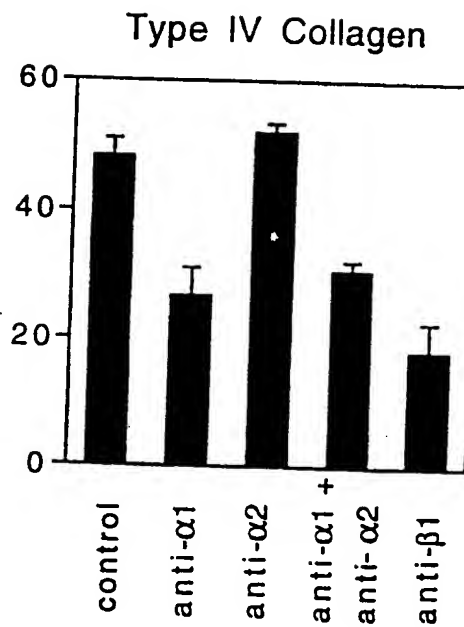


Fig. 6B

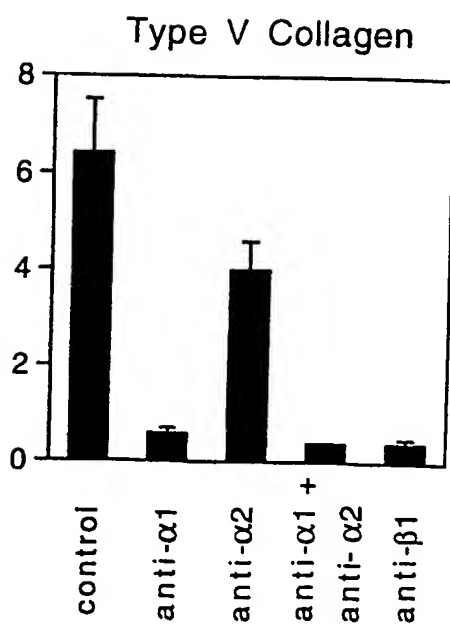


Fig. 6C

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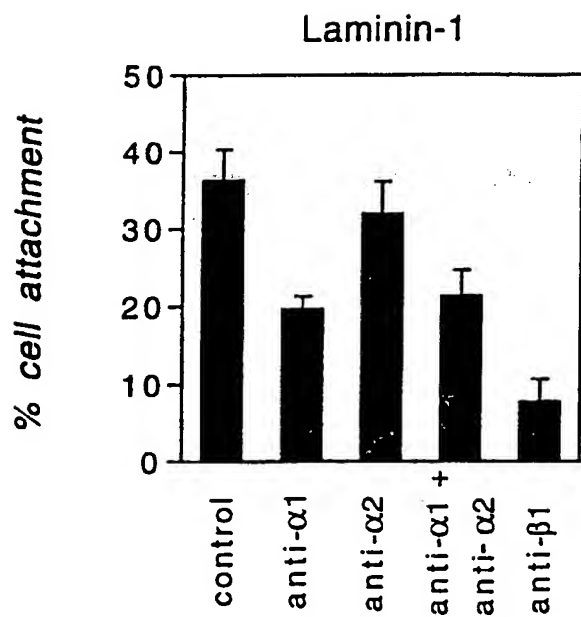


Fig. 6D

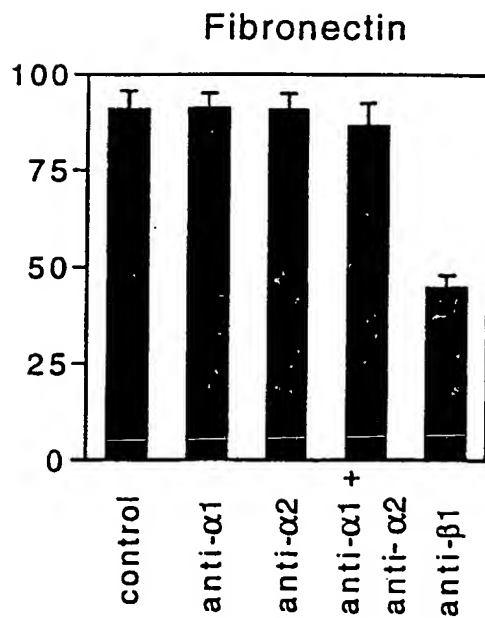
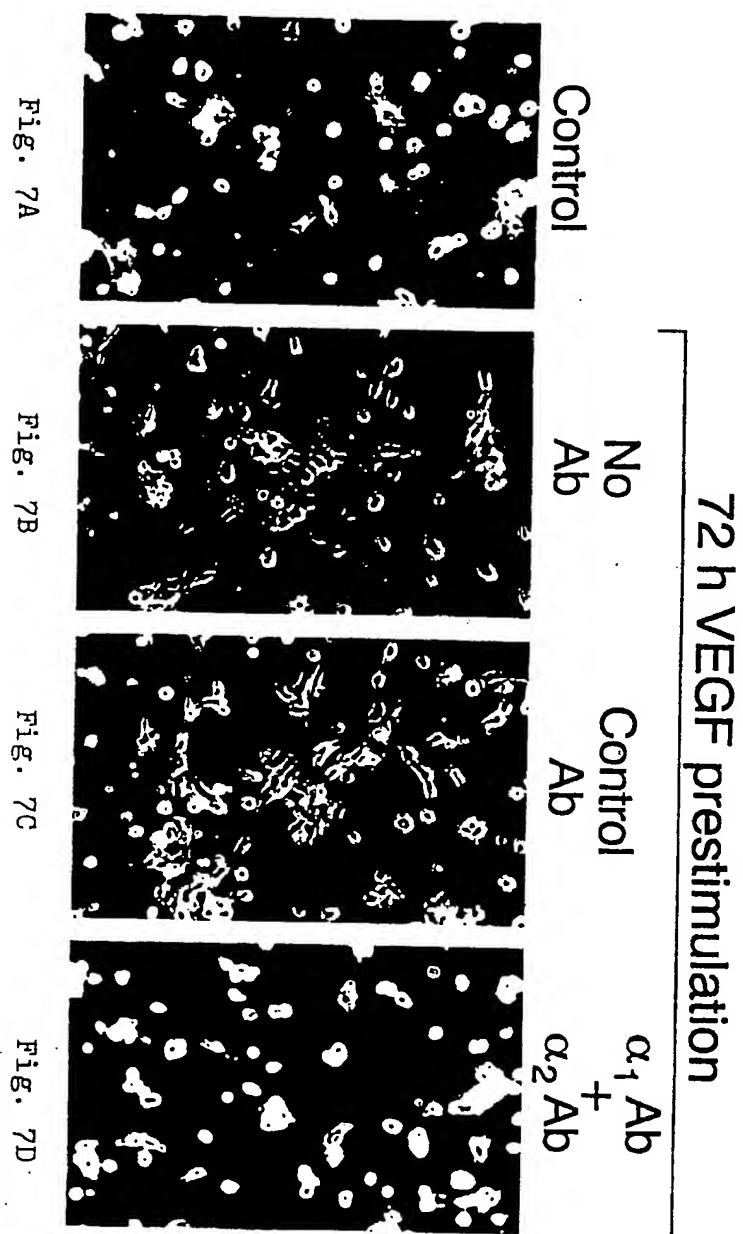


Fig. 6E

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Fig. 8A

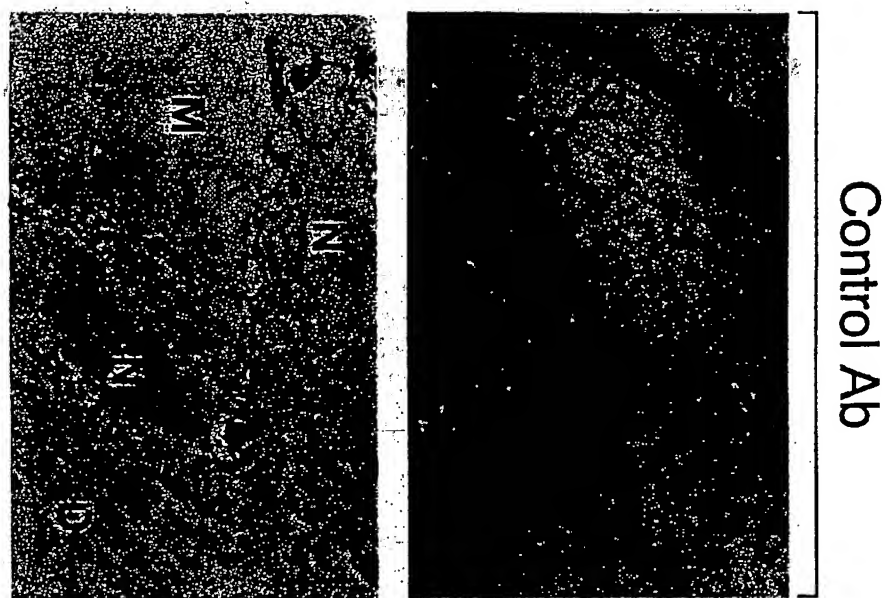


Fig. 8C

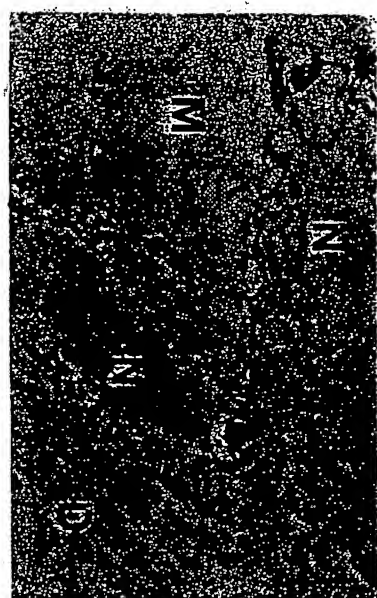


Fig. 8B

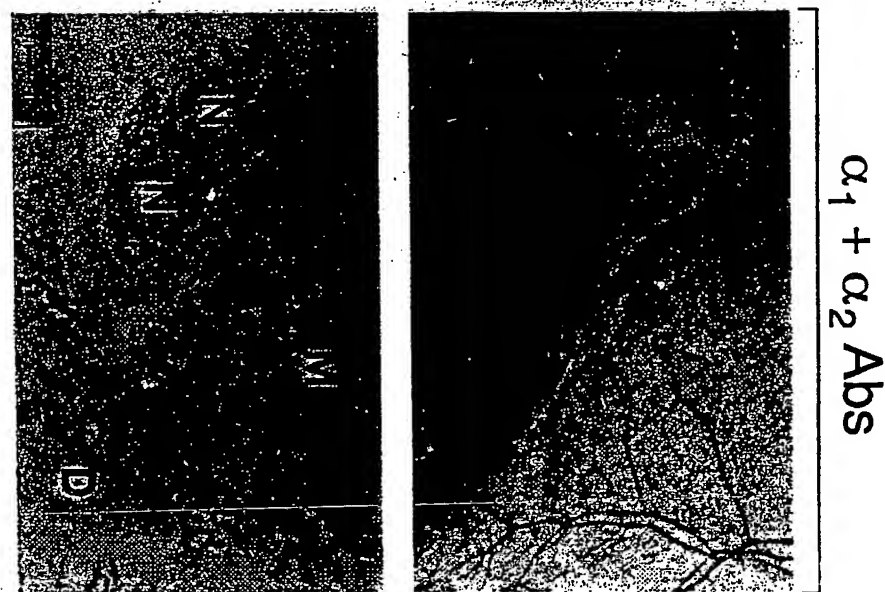
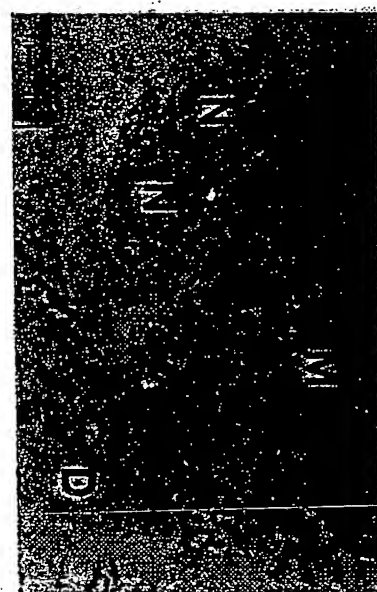


Fig. 8D



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/17485**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/00, 38/18

US CL :424/198.1, 143.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1, 143.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, Biosis, Cancerlit, Embase, Scisearch, WPIDS

search terms: anti-integrin antibody, alpha-integrin, VEGF, treatment of tumours

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WARREN et al. Regulation by Vascular Endothelial Growth Factor of Human Colon Cancer Tumorigenesis in a Mouse Model of Experimental Liver Metastasis. Journal of Clin. Invest., Volume 95, April 1995, see entire document.	1-6
Y	VINK et al. Role of beta-1 integrin in Organ Specific Adhesion of Melanoma Cells in Vitro. Lab. Inves. February 1993. Vol 68. No. 2. pages 192-203, see abstract.	1-6
Y	BASSON et al. Human Enterocyte migration is Modulated in Vitro by Extracellular Matrix Composition and Epidermal Growth Factor. J. Clin. Invest. July 1992. Vol 90. No. 1. pages 15-23, see abstract.	1-6

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 05 DECEMBER 1997	Date of mailing of the international search report 10 FEB 1998
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